THE RELEVANCE OF METHYLATION FOR THIOPURINE CYTOTOXICITY

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THE RELEVANCE OF METHYLATION FOR THIOPURINE CYTOTOXICITY

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Abbreviations

6MP	6-mercaptopurine
PDNS	purine de novo synthesis
IMP	inosine monophosphate
AMP, ATP	adenosine monophosphate, -triphosphate
GMP, GTP	guanosine monophosphate, -triphosphate
AICAR	amidoimidazole carboxamide ribonucleoside
AIC	amidoimidazole carboxamide
Me-MPR	methylmercaptopurine ribonucleoside
PRPP	phosphoribosyl pyrophosphate
PRA	phosphoribosyl amide
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
IMPDH	inosine monophosphate dehydrogenase
PRPPS	phosphoribosyl pyrophosphate sythetase
MTX	methotrexate
tIMP	thio-inosine monophosphate
tGMP	thio-guanosine monophosphate
tGTP	thio-guanosine triphosphate
Me-tIMP	methylthio-inosine monophosphate
Me-tIDP	methylthio-inosine diphosphate
Me-tITP	methylthio-inosine triphosphate
GMPS	guanosine monophosphate synthetase
AdoMet	S-adenosylmethionine
AdoHcy	S-adenosylhomocysteine
TPMT	thiopurine methyltransferase
ALL	acute lymphoblastic leukemia
MPA	mycophenolic acid
HPLC	high performance liquid chromatography
DTT	dithiotreitol
Guo	guanosine
Ado	adenosine
Ade	adenine
Ino	inosine
PCA	perchloric acid
AK	adenosine kinase
R-5-P	ribose-5'-phosphate

CHAPTER 1

INTRODUCTION

Introduction

1.1 General introduction

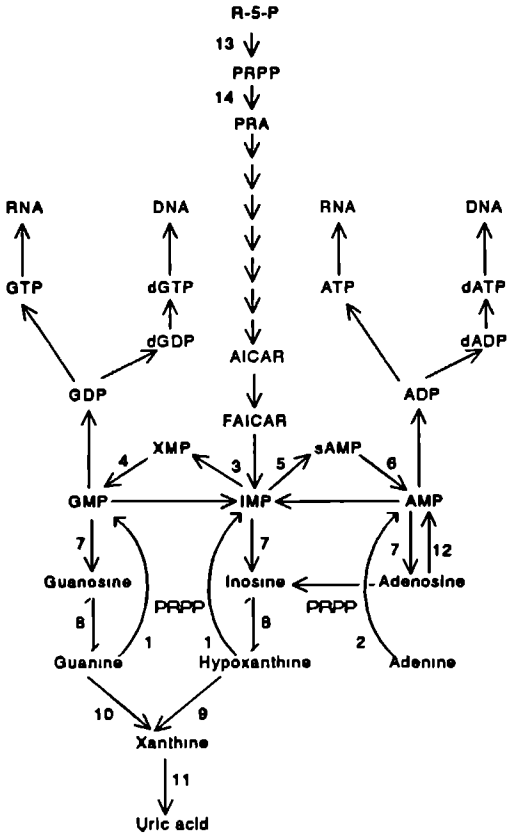
Extensive studies have been performed on the development of anticancer drugs. Many different compounds have been shown to exhibit anticancer activity through different mechanisms. Anticancer drugs often exhibit their activity by using specific biochemical properties of the tumor cells. In general, tumor cells have an accelerated metabolism as compared to their normal counterparts in accordance with their higher cell growth and proliferation rate (1-7). Most anabolic routes are enhanced in tumor cells, whereas catabolic routes are less active (8-11). This imbalance increases with the malignant state of the tumor cells (2,3,8, 10,11) and can be used as a tool for cancer therapy. Drugs, which resemble natural occurring cellular metabolites, can be used to deregulate tumor cell metabolism in order to induce cytotoxicity. These drugs are called antimetabolite. Several metabolic pathways can be used as targets for therapy with antimetabolite. One of these routes is the purine nucleotide synthesis, in which for instance 6-mercaptopurine (6MP) exerts its anticancer activity.

1.2 Purine metabolism

Purine nucleotides are biosynthesized by the purine de novo synthesis (PDNS, Scheme 1) and are required for RNA and DNA synthesis (12). In a process involving eleven reactions the purine ring is built, starting from ribose 5'-phosphate (R-5-P) and ending with the formation of IMP. IMP is converted into AMP or GMP by the purine interconversion pathways. A total of seven high-energy phosphate molecules is required for the ultimate formation of one molecule AMP. Eight high-energy phosphates are necessary for the formation of GMP. So, biosynthesis of purine nucleotides is an energy consuming pathway. Nucleosidemonophosphate kinases and nucleosidediphosphate kinases catalyze the phosphorylation of GMP and AMP into GTP and ATP, respectively. These reactions require ATP (12,13). The activity of the PDNS is regulated by feedback-inhibition by the purine nucleotides. These nucleotides inhibit the second enzyme in the PDNS, phosphoribosyl pyrophosphate amidotransferase, which catalyses the conversion of PRPP into phosphoribosylamine (PRA) (14). The purine interconversion pathway is also regulated by feedback-inhibition, since GMP inhibits IMP dehydrogenase, and AMP inhibits adenylo-succinate synthetase. Furthermore, reciprocal regulation of GMP and AMP synthesis occurs, since ATP is a co-factor for the synthesis of GMP, and GTP for AMP synthesis (12).

Apart from being involved in RNA and DNA synthesis, purine nucleotides are used for many purposes. ATP functions as energy donor for numerous cellular processes (13). GTP can also function as energy donor. Moreover, guanine nucleotides play an important role in

Scheme 1. Purine metabolism



(1) hypoxanthine-guanine phosphoribosyltransferase (HGPRT); (2) adenine phosphoribosyltransferase(APRT); (3) inosinic monophosphate dehydrogenase (IMPDH); (4) guanosine monophosphate synthetase (GMPS); (5) adenylosuccinate synthetase; (6) adenylosuccinate lyase;
(7) purine 5'-nucleotidase (5'NT); (8) purine nucleoside phosphorylase (PNP); (9),(10),(11) xanthine oxidase; (12) adenosine kinase (AK); (13) phosphoribosyl pyrophosphate (PRPP) synthetase (PRPPS);
(14) phosphoribosyl pyrophosphate amidotransferase.

various anabolic processes, such as protein synthesis, intracellular cell signaling, polyamine synthesis and microtubule assembly (15-17).

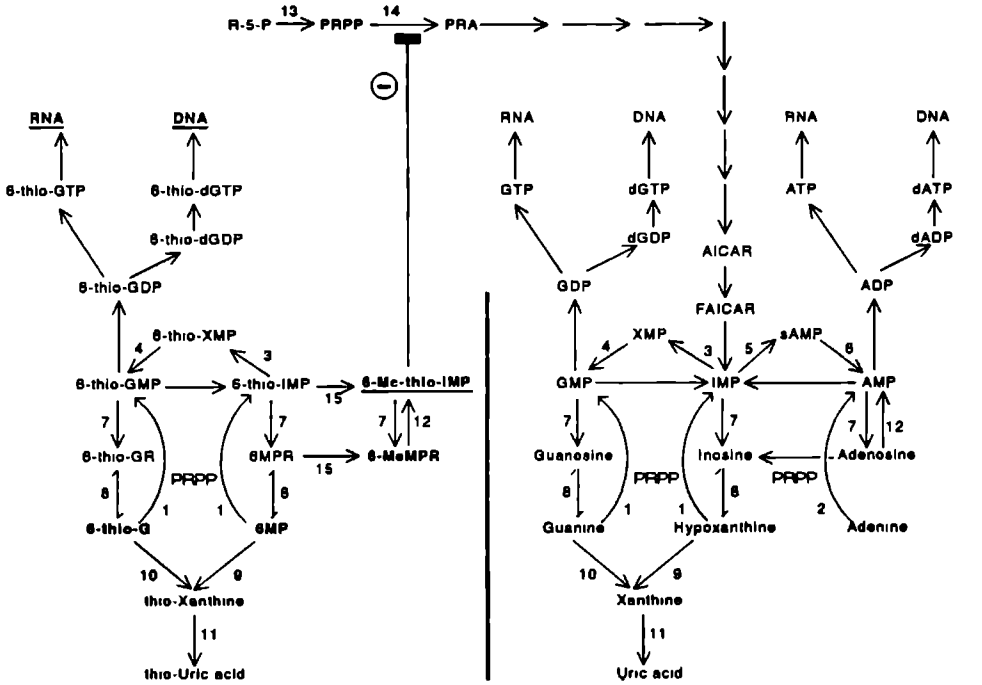
Products of purine nucleotide catabolism are adenosine, guanine and hypoxanthine. Adenosine is converted into inosine and then into hypoxanthine. Guanine is degraded into xanthine. Hypoxanthine and xanthine are degraded further by xanthine oxidase into uric acid, which is the end product of purine catabolism in man. This degradation of purine bases can be prevented by the purine salvage pathway (Scheme 1). In this way, the loss of the purine ring, which is energetically expensive to build, can be prevented (12,18). Hypoxanthine and guanine are converted into IMP and GMP, respectively, by hypoxanthine-guanine phosphoribosyltransferase. Cleavage of 5'-deoxy-5'-methylthio-adenosine (the nucleoside end product of polyamine synthesis) into 5'-methylthioriboside-1-phosphate results in formation of adenine, which is salvaged into AMP by adenine phosphoribosyltransferase. All these reactions use PRPP as a co-substrate.
In most cells the main route of purine formation is the purine salvage pathway (3,19). In malignant lymphoblasts purine salvage and especially purine de novo synthesis exhibit a higher activity as compared to peripheral blood lymphocytes (2,4,8-11,19-21).

1.3 Mechanisms of 6-mercaptopurine cytotoxicity

6MP is an analogue of the natural purine base hypoxanthine and is metabolized by a similar metabolic route. It was first synthesized in 1952, by Elion and Hitchings (22). Soon thereafter, its antileukemic activity was reported by Burchenal et al. (23). 6MP in combination with methotrexate (MTX) is the cornerstone of the maintenance treatment of children with acute lymphoblastic leukemia (ALL) (24). Combination of these two drugs improves therapy results as compared to either drug alone, as demonstrated in earlier clinical trials (25) and biochemically in vitro in our laboratory (26,27). During maintenance treatment both 6MP and MTX are administered orally in a low dose. In most regiments 6MP is given daily, whereas MTX is administered once a week (24). However, after the introduction of intravenous administration of MTX and the biochemical evidence for a potential synergism between 6MP and MTX (26,27), both drugs have also been used intravenously with good clinical results (28). At this moment an extensive pharmacokinetic and pharmacological study is undertaken in the Dutch Childhood Leukemia Study Group protocol ALL-8, using intravenous MTX and 6MP during consolidation therapy.

The difference between 6MP and its analog hypoxanthine is the presence of a mercapto-(SH) group, instead of a hydroxy-(OH) group (Fig. 1) at the 6th position of the purine ring.

Scheme 2. Metabolism of 6-mercaptopurine

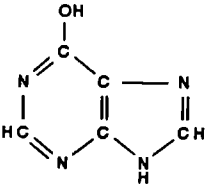


The numbers of the enzymes correspond to the numbers at scheme 1; (15) thiopurine methyltransferase (TPMT)

6MP enters the cells by facilitated transport by the hypoxanthine carrier (29). 6MP is not cytotoxic by itself, but has to be converted in order to exert cytotoxic activity. The first step is conversion into thioIMP (tIMP) by the purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (30,31) (Scheme 2). tIMP can be further metabolized by two pathways.

First, tIMP can be converted by the purine interconversion into thio-GMP (tGMP) (Scheme 2). The enzymes catalyzing this metabolic conversion are IMP dehydrogenase (IMPDH) and GMP synthetase (GMPS). IMPDH is the rate-limiting enzyme in this route (1) and as mentioned above, this enzyme is subject to end product inhibition by GMP. tGMP is converted further by GMP and GDP kinase and ribonucleotide reductase, to form tGTP and

Fig. 1. Structural formula of hypoxanthine and its analogue 6-mercaptopurine.



hypoxanthine 6-mercaptopurine

deoxytGTP, which are incorporated into RNA and DNA of the cells, respectively (27,32-44). Incorporation into DNA is the most important one of these routes (27,33,36,37), inducing DNA damage, such as single strand breaks (38-40,43,44), DNA-protein cross-links (41,43), interstrand cross-links (43) and sister chromatid exchanges (42,43). This results in delayed cytotoxicity in most tumor cell lines and is commonly thought to be the main cause for 6MP cytotoxicity (37,39,44-48). This process of delayed cytotoxicity is reflected by growth arrest of the tumor cells in the G_2 + M phase of the cell cycle (36,46).
The second route of tIMP metabolism is methylation by thiopurine methyltransferase into methyl-thioIMP (Me-tIMP, Scheme 2) (49). The predominant route of Me-tIMP formation appears to be via methylation of tIMP (50). This conversion is S-adenosylmethionine (AdoMet) dependent (50-54). Me-tIMP is a very strong inhibitor of the PDNS (14,55-57). Inhibition occurs at the second enzyme in this route, PRPP amidotransferase, catalyzing conversion of PRPP into PRA (14,50,52,58-62). Next to Me-tIMP tIMP also inhibits PDNS, but Me-tIMP is the most effective of the two (50). Inhibition of PDNS can provoke cytotoxicity by several different mechanisms:

1. Inhibition of PRPP amidotransferase results in accumulation of PRPP (36,45,63,64). Since PRPP is a co-substrate for the intracellular incorporation and conversion of 6MP, an increase in PRPP concentration induces an increase in the incorporation of 6MP into the cells and autostimulation of 6MP cytotoxicity occurs (36,52,65-67).

2. Inhibition of the PDNS by Me-tIMP results in a depletion of endogenous purine nucleotides (36,58,61,62,68). Purine nucleotides are involved in many metabolic processes, as described earlier. Tumor cells have a high need of purine metabolites for cell growth and proliferation. Depletion of these purine nucleotides results in inhibition of RNA and DNA synthesis (36) and may inhibit many other purine nucleotide dependent processes in the cells. This results in growth inhibition of the tumor cells in late G_1 + S phase of the cell cycle and induces inhibition of cell growth and, after high dose or prolonged administra-tion

of 6MP, cytotoxicity (36,62,69).

3. The excess of PRPP, which results from inhibition of PDNS at PRPP amidotransferase, induces an increase of pyrimidine biosynthesis, since the PRPP concentration regulates this pathway (47,61,62,68,70-72). Taken together with the decrease of the purine concentrations, the increase of pyrimidine nucleotides may lead to unbalanced growth, which can also result in cell growth inhibition.

Besides these mechanisms of 6MP cytotoxicity, metabolites of 6MP are known to interfere with several other enzymes of purine metabolism. For instance, high concentrations of tIMP inhibit IMPDH, the rate-limiting enzyme of the purine interconversion pathway involved in guanine nucleotide biosynthesis (52,60,73-76). tIMP also inhibits GMPS, the other purine interconversion enzyme involved in guanine nucleotide biosynthesis (75). At high concentrations, tIMP may inhibit both adenylosuccinate synthetase and adenylosuccinate lyase, the enzymes catalyzing the conversion of IMP into AMP (52,60,75-77). Furthermore, as mentioned before, tIMP inhibits PDNS, although not as potent as Me-tIMP. tGMP inhibits IMPDH, as well as GMP kinase, PRPP amidotransferase and HGPRT (47).

The importance of the methylation route for 6MP cytotoxicity has yet not fully been understood. The metabolism of Me-tIMP can be studied more specifically by using methylmercaptopurine ribonucleoside (Me-MPR), another thiopurine antimetabolite of which Me-tIMP is a metabolite. Transfer of cells which are treated with Me-MPR into drug-free and purine rich medium results in reversal of the growth-inhibitory activity of Me-MPR and a decrease of the concentration of Me-tIMP in the cells (61). The reversal of growth inhibition can be ascribed to repletion of purine nucleotides as a result of salvage of the freshly added purine nucleotide precursors, present in the drug-free medium. But it may also be caused by the decrease of intracellular Me-tIMP concentrations, observed after the transfer of the cells into the drug-free medium. This decrease may indicate conversion of Me-tIMP into methylmercaptopurine ribonucleoside (Me-MPR), followed by extracellular excretion (61). However, other studies indicated that Me-tIMP concentrations diminished only slowly (78,79). Demethylation of Me-tIMP is reported to occur in vivo in the rat. Until now the enzyme catalyzing this reaction has been found exclusively in the liver (65), so this catabolic pathway for Me-tIMP seems to be of no importance in leukemia cells. Furthermore, it is generally assumed that phosphorylation of Me-tIMP does not occur (56,80,81). However, two reports have mentioned formation of Me-tIDP and Me-tITP in human peripheral blood leukocytes and nucleated marrow cells in vitro and in patients receiving 6MP treatment (82,83), so this statement remains to be elucidated. Our previous studies did not give any indication of the formation of these metabolites. Overall, the metabolic fate of

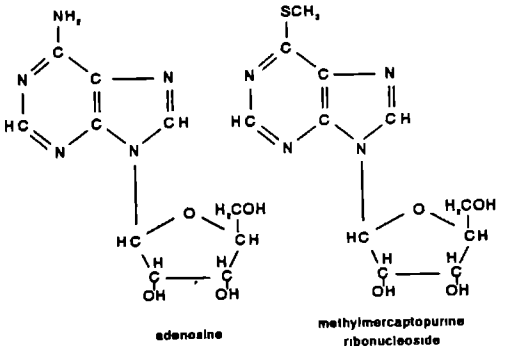
Me-tIMP is not yet fully elucidated.
A distinction between inhibition of PDNS and cytotoxicity of 6MP was made by Tidd et al. (45). They concluded that inhibition of PDNS was not centrally involved in the delayed cytotoxic activity of 6MP, but that 6MP cytotoxicity rather depended on incorporation of thioguanine nucleotides into DNA. This conclusion was based on the observation that exposure of L5178Y mouse lymphoma cells to Me-MPR resulted in depletion of purine nucleotide concentrations and cell proliferation, but that it did not affect cell viability. After Me-MPR was removed, cell proliferation returned to control values. In contrast, a brief exposure of these cells to 6MP had a slight effect on the cellular purine nucleotide concentrations, but it did induce a delayed cytotoxic reaction, as a result of the incorporation of thioguanine nucleotides into the DNA.

Similar conflicting ideas also exist with respect to the clinical relevance of the methylation route of 6MP. Wide interindividual differences exist in the activity of TPMT, as a result of a common genetic polymorphism (53). Overall, 1 in 300 normal subjects exhibits undetectable TPMT activity, 11 percent of the population has intermediate activity and the remainder of the population high activity (53). A reciprocal correlation between TPMT activity and formation of thioguanine nucleotides exists (84-86). In patients who received oral 6MP treatment and who developed a severe leukocytopenia as a result of the treatment, low TPMT activities were measured. These low actvities led to high concentrations of thioguanine nucleotides in red blood cells, correlated with toxicity (86,87). Furthermore, children with ALL who exhibited a high TPMT activity in red blood cells had a poorer prognosis as compared with patients with an intermediate TPMT activity in red blood cells after 6MP treatment (88). In this view, methylation is considered to be a detoxification pathway for 6MP.
On the other hand, however, it was reported that the concentration of 6MP required to induce a 50 % inhibition of DNA synthesis in phytohemagglutinin stimulated peripheral blood lymphocytes was higher in subjects with a genetically low TPMT activity compared to subjects with genetically normal or high TPMT activity. This seems to indicate that methylated metabolites of 6MP are important for cytotoxicity (85). So, the clinical relevance of the methylation route for 6MP cytotoxicity is far from clear and is one of the subjects of the ALL-8 study of the Dutch Childhood Leukemia Study Group, using intravenous 6MP in children with ALL.

1.4 Mechanisms of methylmercaptopurine ribonucleoside cytotoxicity

Methylmercaptopurine ribonucleoside (Me-MPR) is another purine antimetabolite. This cytostatic drug is an analogue of the purine nucleoside adenosine (Fig. 2). Me-MPR induces inhibition of cell growth and cell death in tumor cells in vitro (61,62,80,89). Furthermore,

Fig. 2. Structural formula of adenosine and its analogue methylmercaptopurine ribonucleoside.



Me-MPR is effective in experimental tumors resistant to 6MP (80), which is probably the result of the fact that the development of cellular resistance to 6MP is commonly associated with the loss of HGPRT activity. HGPRT converts 6MP into its cytotoxic form, tIMP (90), whereas Me-MPR is conversed by adenosine kinase (AK) into Me-tIMP (56,65,80,91-93) (Scheme 3). As discussed above, Me-tIMP inhibits PDNS, leading to inhibition of cell growth and cytotoxicity. Synergism is observed between 6MP and Me-MPR (63,64). This synergism is based on the same mechanism as the autostimulation of 6MP cytotoxicity described earlier. It can be ascribed to an increase of the PRPP concentration as a result of the inhibition of PDNS by Me-tIMP. As a result more 6MP can be incorporated into the cells by HGPRT, since PRPP is the co-substrate for this conversion, and an increase in cytotoxicity results.

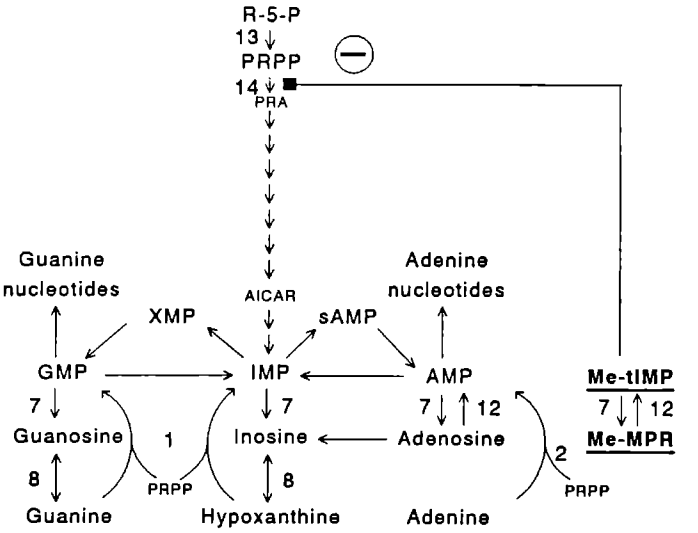
1.5 Aim of the present study

In our laboratory extensive studies have been performed to elucidate the mechanisms of cytotoxicity of 6MP (94,95). From these studies a possible role for formation of Me-tIMP for 6MP cytotoxicity emerged. This was in contrast with the studies of others, who regarded Me-tIMP formation as detoxification pathway (85-88).

In this study special attention is paid to the importance of the methylation route for 6MP cytotoxicity. The effects of high dose versus low dose 6MP are evaluated in a human malignant T-lymphoblastic cell line, Molt F4. It is especially important to gain more insight in the mechanisms of cytotoxicity of high dose 6MP, because at this moment clinical trials with intravenously administered 6MP are performed. As a result, much higher plasma concentrations of 6MP are reached as compared with oral administration.

Molt F4 cells have a highly active purine salvage and PDNS (26), so 6MP can be easily incorporated and can fully exert its effects on DNA, RNA and PDNS.

Scheme 3. Metabolism of methylmercaptopurine ribonucleoside



The numbers of the enzymes correspond to those of schemes 1 and 2.

To obtain a better insight in the contribution of Me-tIMP to 6MP cytotoxicity, conversion of tIMP into tGMP was prevented in some studies by inhibition of IMPDH. In that way the effects of 6MP via the methylation route could be studied specifically. Inhibition of IMPDH was obtained by mycophenolic acid (MPA), a well known and specific inhibitor of IMPDH (96,97).

In chapter 2 the development of a sensitive micro-assay for the measurement of IMPDH activity and the effects of MPA on IMPDH activity are described.
In chapter 3 the effects of inhibition of IMPDH on cell growth and cell viability are described. Endogenous nucleotide concentrations have been determined, to obtain insight in the MPA-effects on purine and pyrimidine metabolism.
Chapter 4 deals with the importance of Me-tIMP formation for cytotoxicity of 6MP after administration of 6MP alone, or in combination with MPA. The effects of addition of both drugs are monitored by determination of cell growth and cell viability and by determination of endogenous nucleotides and metabolites of 6MP.
In chapter 5 the effects of Me-tIMP formation on cellular cytotoxicity are studied in more

detail by the use of Me-MPR, since Me-tIMP is the sole metabolite of Me-MPR. In this chapter the effects of several concentrations of Me-tIMP on cytotoxicity and inhibition of PDNS are established, the latter being determined by measuring both the increase of PRPP concentration and the decrease of endogenous purine nucleotides.
The importance of Me-tIMP formation and subsequent inhibition of PDNS were further evaluated in the chapters 6 and 7. In chapter 6 experiments are described in which 6MP is combined with amidoimidazolecarboxamide ribonucleoside (AICAR). AICAR is an intermediate of the purine de novo synthesis, distal to the PDNS inhibition site of Me-tIMP. Therefore, AICAR is able to circumvent this inhibition. Since Me-MPR is also converted into Me-tIMP, the effects of the combination of Me-MPR and AICAR are also described.
Chapter 7 deals with prevention of Me-MPR cytotoxicity by intermediates of the purine salvage pathway adenosine, adenine, inosine and guanosine, and evaluates the importance of the different nucleotide pools for Me-tIMP cytotoxicity.
Finally, a new mechanism by which the methylation pathway of 6MP can influence cell proliferation is proposed in chapter 8. As a result of 6MP treatment and methylation of tIMP a decrease in the S-adenosyl homocysteine (AdoMet) concentration occurs. Since AdoMet is a universal methyl-donor involved in methylation of many different intracellular molecules, for instance proteins, lipids and nucleic acid, a decrease of its concentration may disrupt cellular processes, leading to inhibition of cell growth and cytotoxicity.

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CHAPTER 2

6-MERCAPTOPURINE METABOLISM IN TWO LEUKEMIC CELL LINES.

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6-Mercaptopurine metabolism in two leukemic cell lines

Introduction

6-Mercaptopurine (6MP), an analogue of the purine base hypoxanthine, is commonly used in the maintenance therapy of acute lymphoblastic leukemia (ALL) in children (1).
The first step of 6MP cytotoxicity is intracellular conversion into thio-IMP (tIMP) by the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT). tIMP can be further metabolized by two pathways. Firstly, it can be converted into thioguanine nucleotides by the purine interconversion route (2,3). Subsequently, these thioguanine nucleotides are incorporated into RNA, and in particulary into DNA (4,5). Incorporation of thioguanine nucl-eotides into DNA results in delayed cytotoxicity, probably due to induction of DNA damage (6,7). The rate limiting enzyme in this metabolic route is IMP dehydrogenase (IMPDH) (8), and as such its activity is of importance for the efficacy of 6MP cytotoxicity. Secondly, tIMP can be methylated by thiopurine methyltransferase (TPMT) (9,10). Methyl-thioIMP (Me-tIMP) formed by this reaction is a strong inhibitor of the purine de novo synthesis (PDNS) (11) and this may also contribute to the cytotoxicity of 6MP by induction of depletion of normal nucleotides for DNA and RNA synthesis.
One of the possibilities to elucidate the role of both metabolic routes in the effectiveness of 6MP therapy is to inhibit either one of them. In this study mycophenolic acid (MPA), a specific inhibitor of IMPDH was used to inhibit the conversion of 6MP into thioguanine nucleotides. A microassay for IMPDH was developed, based on the assay des-cribed by Uchida et al (2). IMPDH activity was determined in two leukemic cell lines, Molt F4 (a T-ALL cell line) and KM_3 (a non-T-non-B cell line). The effects of MPA on cell growth, cell viability, IMPDH activity and cellular nucleotide concentration were determined.

Materials and methods

6MP was purchased from Wellcome bv, Netherlands, MPA from Sigma Chemicals, USA, and ^{14}C-IMP from Amersham international, UK.

Cell culture

The experiments were performed with Molt F4, a T-ALL cell line, and with KM_3, a non-T-non-B cell line. The cells were cultured in RPMI 1640 DM (Gibco, Netherlands), supplemented with 10 % non-dialyzed fetal calf serum (Gibco, Netherlands), 2 mM L-glutamine (Sigma Chemicals, USA), 2 mM sodium pyruvate (BDH Chemicals Ltd, UK) and gentamicin/streptomycin, thrice a week. The cell cultures were maintained in a 5 % CO_2 humified atmosphere at 37°C. The absence of mycoplasma contamination and the presence of marker antigens were tested regularly.

IMPDH assay

For determination of the IMPDH activity a microassay was developed, based on the assay described by Uchida et al (2). Cells were lyophilized overnight and were incubated with 1.61 μmol Tris/HCl (Sigma Chemicals, USA) pH 8.0, 12.9 nmol reduced glutathione (GSH, sigma Chemicals, USA), 3.23 nmol EDTA (Siegfried AG, BDR), 3.23 nmol NAD^+ (Sigma Chemicals, USA), 0.16 μmol KCl (Merck, BDR) and 2.9 nmol [8-^{14}C]IMP, for 60 minutes at 37°C. The reaction volume was 10 μl. Substrate and product formed were separated by means of thin layer chromatography. The radioactivity was counted in a liquid scintillation counter (Delta, Netherlands). The specific activity (SA) of the enzyme was expressed as nmoles product.10^{-6} viable cells.h^{-1}.

The effect of inhibition of IMPDH activity by MPA was studied in cell lysates.

Determination of endogenous nucleotides

After cell culture during 4 hours in the presence of various concentrations of MPA the cells were harvested, counted in a coulter counter and cell viability was determined. Nucleotides were extracted from 3 x 10^6 viable cells and stored at -20°C until analysis. Nucleotide concentrations were determined by means of HPLC (12).

Cell growth and viability assays

Cell growth and viability experiments were performed in logarithmically growing cells, which were seeded 24 hours prior to the start of the experiment. At this time 6MP, MPA or a combination of both were added as a single dose. During the experiment 2 mM glutamine was supplemented every 24 hour, in order to prevent glutamine exhaustion of the medium. Cells were harvested and counted in a coulter counter. Cell viability was determined by the trypan blue exclusion.

Results

The specific activity of IMPDH was higher in Molt F4 cells as compared to KM_3 cells (1.9 $\pm$ 0.2 nmoles product.10^{-6} viable cells.h^{-1} (mean $\pm$ SEM), n = 60 versus 1.1 $\pm$ 0.1, n = 11). MPA appeared to be a strong inhibitor of IMPDH (Fig. 1). The inhibition was concentration de-pendent. A fifty percent inhibition of the enzyme activity was observed in the presence of 0.2 μM MPA. With 5 μM MPA virtually no IMPDH activity could be detected.

The inhibition of IMPDH by MPA was reflected by a decrease of intracellular guanine nucleotide levels (Fig. 2). When cells were incubated during 4 hours with 0.8 μM MPA, a fifty percent reduction of the guanine nucleotide level was observed.

Both 6MP and MPA were cytotoxic for Molt F4 cells (Fig. 3a, 3b). The effects of the drugs were more pronounced on cell growth (fig. 3a) than on cell viability. The

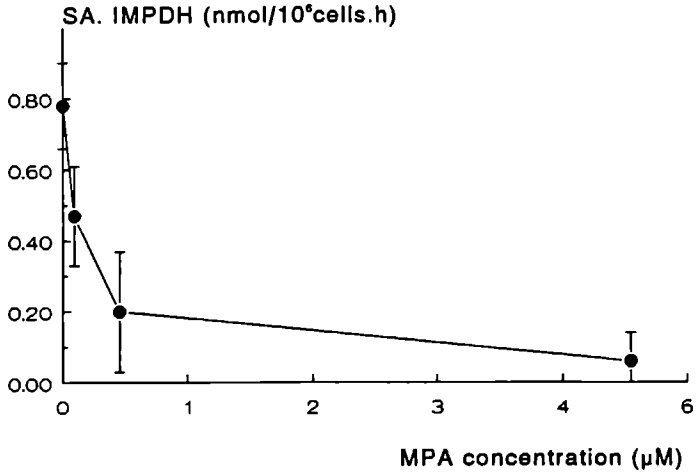

Fig. 1. Inhibition of IMPDH activity (mean ± SD, n=15) from Molt F4 cells by MPA. The IMPDH activity was determined in lysates of 25,000 Molt F4 cells in the presence of various concentrations of MPA.

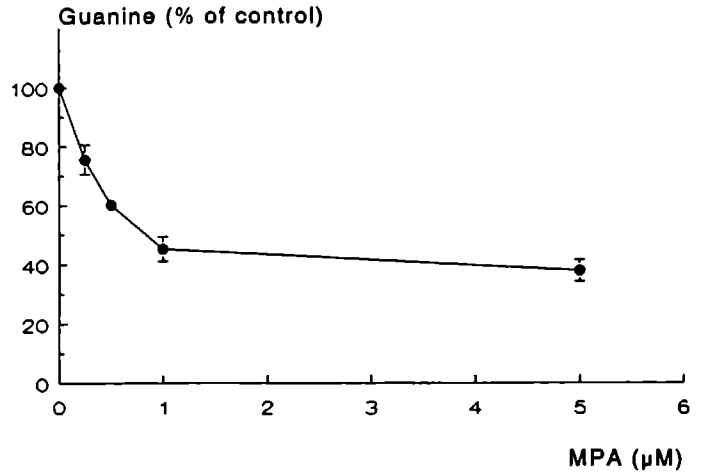

Fig. 2. Effect of MPA on the cellular guanine nucleotide concentration of Molt F4 cells. The cells were incubated in various concentrations of MPA for 4 hours (mean ± SD, n=3).

remaining viability after an incubation with 2 μM 6MP during 48 hours was 80 % of control. Cell growth, however, was decreased to 45 % of control. The combination of 6MP and MPA resulted in a synergistic decrease of cell growth and cell viability (Fig. 3a,b).

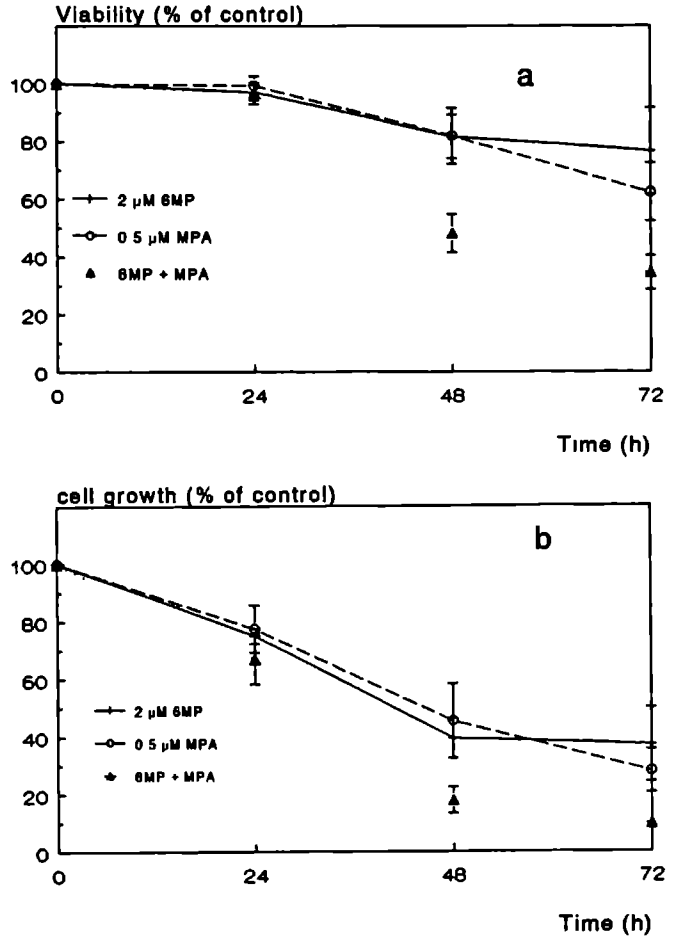

Fig. 3. Effects of 2 µM 6MP, 0.5 µM MPA and the combination of both drugs on cell viability (a) and cell growth (b) of Molt F4 cells. Viability and cell growth were expressed as percentage of untreated cells (mean ± SD, n=5).

Discussion

The results of this study show that IMPDH activity is higher in Molt F4 cells than in KM_3 cells. Earlier studies in our laboratory demonstrated that Molt F4 cells also exhibit a more active PDNS and purine salvage pathway activity (13). These observations indicate that the purine flow to guanine nucleotides is higher in Molt F4 cells than in KM_3 cells.

MPA inhibits IMPDH in a concentration dependent manner (Fig. 1). The inhibition of IMPDH is reflected by a decrease of intracellular guanine nucleotide levels (Fig. 2). The

guanine nucleotide concentration decreases to 40 % of control values when cells are incubated during 4 hours with 5 μM MPA. These results are in accordance with data from earlier studies (14). The inhibition of IMPDH, however, in the presence of 5 μM MPA, is much more pronounced than indicated by the guanine nucleotide concentration; hardly any IMPDH activity is present (Fig. 1). The presence of measurable guanine nucleotide levels after 4 hours may reflect the production of guanine nucleotides by the salvage pathway.

MPA is cytotoxic for Molt F4 cells. Cytotoxicity may be the result of induction of depletion of guanine nucleotides. Firstly, depletion of guanine nucleotides may inhibit DNA and RNA synthesis. Secondly, this depletion could hamper intracellular signal transduction, as guanine nucleotide binding proteins (G-proteins) are often involved in this process (15,16). Alternatively, the observed cytotoxicity can be the result of increased IMP, which is an inhibitor of PDNS (11). Inhibition of purine synthesis may lead to decreased DNA and RNA synthesis, resulting in inhibition of cell growth and cell viability, in particulary because Molt F4 cells depend on a high PDNS activity (13).

6MP cytotoxicity can be caused by incorporation of thioguanine nucleotides into DNA and RNA and by formation of Me-tIMP, an inhibitor of PDNS. This study shows that methylation of tIMP is an important route for 6MP cytotoxicity. This can be concluded from the observed synergistic effect of the combination of 6MP and MPA on the cell viability. MPA inhibits IMPDH and thereby the conversion of 6MP into guanine nucleotides. The methylation of tIMP into Me-tIMP by TPMT remains the only route available. Also, a substantial quan-tity of Me-tIMP could be detected when cells were grown in the presence of 6MP (results not shown). Me-tIMP is a strong inhibitor of the PDNS (11). As the PDNS is inhibited, the intracellular PRPP concentration rises (13). PRPP is a co-substrate needed for the conversion of 6MP into tIMP. So, as a result of the increase of the PRPP concentration more 6MP can be converted into tIMP. In this way, a auto-enhancement of 6MP incorporation may occur.

Since 6MP and MPA demonstrated a severe inhibition of cell growth when added separately, no conclusion can be drawn about a synergistic effect of the combination of both drugs on cell growth.

In these experiments the effects of 6MP and MPA on cell growth can be observed at an earlier stage and are more pronounced than the effects on cell viability. First cell growth inhibition is induced by the inhibition of intracellular metabolic activity caused by 6MP and MPA. Then, as a result of this, cell death occurs. So, biochemical changes occur before cell death can be detected.

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CHAPTER 3

INHIBITION OF IMP DEHYDROGENASE BY MYCOPHENOLIC ACID IN MOLT F4 HUMAN MALIGNANT LYMPHOBLASTS.

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Inhibition of IMP dehydrogenase by mycophenolic acid in Molt F4 human malignant lymphoblasts

Summary

The effects of inhibition of IMP dehydrogenase (IMPDH), the rate-limiting enzyme in guanine nucleotide de novo synthesis, on cell growth, cell viability, endogenous nucleotide concentrations and concentrations of extracellular nucleosides and bases were studied in Molt F4 human malignant lymphoblasts. Mycophenolic acid (MPA) was used as a specific inhibitor of the enzyme activity. IMPDH activity was maximally inhibited with 0.5 μM MPA. After a 2 h exposure of the cells to 0.5 μM MPA, guanine nucleotides were depleted to approximately 50 % of control values, whereas 5-phosphoribosyl-1-pyrophosphate (PRPP) levels increased to approximately 200 %. Under these conditions, cytotoxicity became obvious after 24 h. Depletion of guanine nucleotides and cytotoxicity were prevented by addition of guanosine to MPA treatment. Daily supplements of guanosine were required to prevent MPA cytotoxicity during the entire incubation period of 72 h. We conclude that depletion of guanine nucleotides, induced by treatment with MPA, induces a severe and rapid cytotoxicity in Molt F4 cells.

Introduction

IMP dehydrogenase (IMPDH) is a key enzyme in purine interconversion. It catalyses the conversion of IMP into XMP and is the rate-limiting enzyme in the de novo synthesis of guanine nucleotides (1). Guanine nucleotides play an important role in various anabolic cellular processes, such as protein synthesis, intracellular cell signalling, polyamine synthesis and microtubule assembly. Furthermore, guanine nucleotides are necessary for RNA and DNA synthesis and for several specific enzyme reactions and function as energy donors for numerous metabolic processes in cells (2-5).

Tumor cells exhibit a high need for nucleotides, as their metabolism is accelerated to facilitate cell proliferation. Therefore, activities of many enzymes controlling purine and pyrimidine metabolism show marked differences in lymphoblasts as compared with lymphocytes (1,6,7). IMPDH is also reported to have a higher activity in tumor cells as compared to normal cells (1,8,9). Two isoenzymes of IMPDH are known, type I and type II, of which type II-mRNA and enzyme activity are upregulated in tumor cells (10,11).

Inhibition of de novo synthesis of guanine nucleotides and subsequent depletion of the guanine nucleotide pool may induce cytotoxicity, especially in tumor cells with their high IMPDH activity. Several inhibitors of IMPDH are known to exhibit an antitumor activity (12-15). One of these inhibitors is mycophenolic acid (MPA), a toxic metabolite produced

by penicillium species (16). MPA is a specific inhibitor of IMPDH (12,17). It binds to the NAD^+ binding site of the enzyme. As a result an enzyme-XMP-MPA complex is formed and IMPDH activity is inhibited (18-20). This inhibition is reversible (19). Treatment of cells with MPA results in inhibition of DNA synthesis and a G_1-S phase block occurs (12,17,21,22) as a result of dGTP depletion. Furthermore, inhibitors of IMPDH induce differentiation of several lymphoblastic cell lines (23,24).
In this study the effects of inhibition of IMPDH were studied in Molt F4, a human T-lymphoblastic cell line. Human malignant lymphoblasts have a highly active purine de novo synthesis and purine salvage pathway (25,26). So, interference with guanine de novo synthesis may have profound effects on cell growth, and cell viability in these cells.

Materials and methods
MPA and guanosine were purchased from Sigma Chemicals, USA. [8-^{14}C]IMP (56 mCi/mmol) was obtained from Amersham International, UK, and [^{14}C-carboxyl] orotic acid (52.5 mCi/mmol) from NEN, USA.

The experiments were performed in Molt F4 cells, a human T-cell acute lymphoblastic leukemia cell line. Cell culture procedures have been described in detail previously (27).
For the determination of IMPDH activity 25,000 Molt F4 cells were required. IMPDH activity was determined by the radiochemical micro-assay method described earlier (28).
Direct inhibition of IMPDH by MPA was determined by pre-incubation experiments. Several amounts of MPA were added to reaction buffer, containing 1.61 μmol Tris/HCl pH 8.0 (Sigma Chemicals USA), 12.9 nmol reduced glutathione (GSH, Sigma Chemicals, USA), 3.23 nmol EDTA (Siegfried S.A., Switzerland), 3.23 nmol NAD^+ (Sigma Chemicals, USA) and 0.16 μmol KCl (Merck, Germany). Two μl of this reaction mixture were added to the lyophilized cell lysates and pre-incubation was performed for 20 minutes at 37°C. Hereafter, 8 μl of the above reaction mixture, but with radiolabelled IMP were added, to a final volume of 10 μl and incubation was performed for 60 minutes at 37°C. The end concentration of [8-^{14}C]IMP was 2.9 nmol. The enzyme activity was determined by the method previously described (28).

To determine cell growth, cell viability and nucleotide concentrations, logarithmically growing cells were seeded in a concentration of 0.2 x 10^6 cells per ml, 24 h prior to the start of the experiments. MPA (0.5 and 1 μM) and guanosine (25 μM) were added as a single dose in a small volume (1/100). Experimental procedures have been described elsewhere (27).
Endogenous nucleotides were extracted from 3 x 10^6 viable cells by means of 0.4 M perchloric acid (PCA, BDH Chemicals Ltd, UK) extraction, and were determined by means of

separation by HPLC and monitored at a wavelength of 254 nm as described earlier (29). The concentrations were expressed as pmoles/10^6 viable cells.
Medium nucleosides and bases were extracted with a final concentration of 0.4 M PCA from 0.5 ml medium and were determined by means of reverse-phase HPLC and monitored at a wavelength of 254 nm. Concentrations were expressed as μmoles/L.

The assay for determination of the PRPP concentration is based on the enzymic conversion of PRPP with [^{14}C-carboxyl] orotic acid to $^{14}CO_2$ and uridine monophosphate and has been described earlier (30).

Results

The specific activity of IMPDH in Molt F4 human lymphoblasts without preincubation was 2.0 $\pm$ 0.2 nmol.10^{-6} viable cells.h^{-1} (mean $\pm$ SEM). As a result of pre-incubation IMPDH activity in the control dropped to 0.9 $\pm$ 0.1 nmol.10^{-6} viable cells.h^{-1}. Pre-incubation of Molt F4 cell lysates with 0.1 μM MPA resulted in a decrease of IMPDH activity to 0.35 $\pm$ 0.1 nmol.10^{-6} viable cells.h^{-1}. Pretreatment with 0.5 μM MPA led to a reduction of enzyme activity to 0.27 $\pm$ 0.2 nmol.10^{-6} viable cells.h^{-1}.
Cytotoxicity of MPA in Molt F4 cells was concentration-dependent (Fig. 1). Treatment of cells with 0.5 μM MPA induced a severe inhibition of cell growth and a 50 % decrease of cell viability at 72 h. Under these conditions intracellular guanine nucleotide concentrations were depleted to approximately 40 % of control values (Table I). With 1 μM MPA inhibition of cell growth and cell viability was more severe (Fig. 1). However, depletion of guanine nucleotides was almost similar (results not shown).
No effect was observed on the intracellular adenine nucleotide concentration as a result of treatment of the cells with 0.5 μM MPA (Table I). Intracellular cytosine and uracil nucleotides increased slightly during the first 6 h after treatment (Table II).
Furthermore, uridine and cytosine (in the form of cytidine) were excreted in the medium as a result of MPA treatment (Table III). The intracellular PRPP concentration increased from 90 pmol/10^6 viable cells in untreated cells to 180 pmol/10^6 viable cells 4 h after treatment with 0.5 μM MPA.
Addition of a single dose of 25 μM guanosine partly prevented inhibition of cell growth by 0.5 μM MPA during the first 24 h of the treatment. Hereafter, inhibition of cell growth still occurred (Fig. 1a). Cell viability remained at control values during the first 48 h of the treatment, but dropped to a value resembling that of cells treated with MPA alone after 72 h (Fig. 1b). This MPA cytotoxicity, occurring despite the addition of guanosine once-only, can be attributed to depletion of intracellular guanine nucleotides, which occurred after 24 h, resulting from exhaustion of guanosine in the culture medium (Table III).
25 μM guanosine had to be supplemented daily to treatment with 0.5 μM MPA to prevent

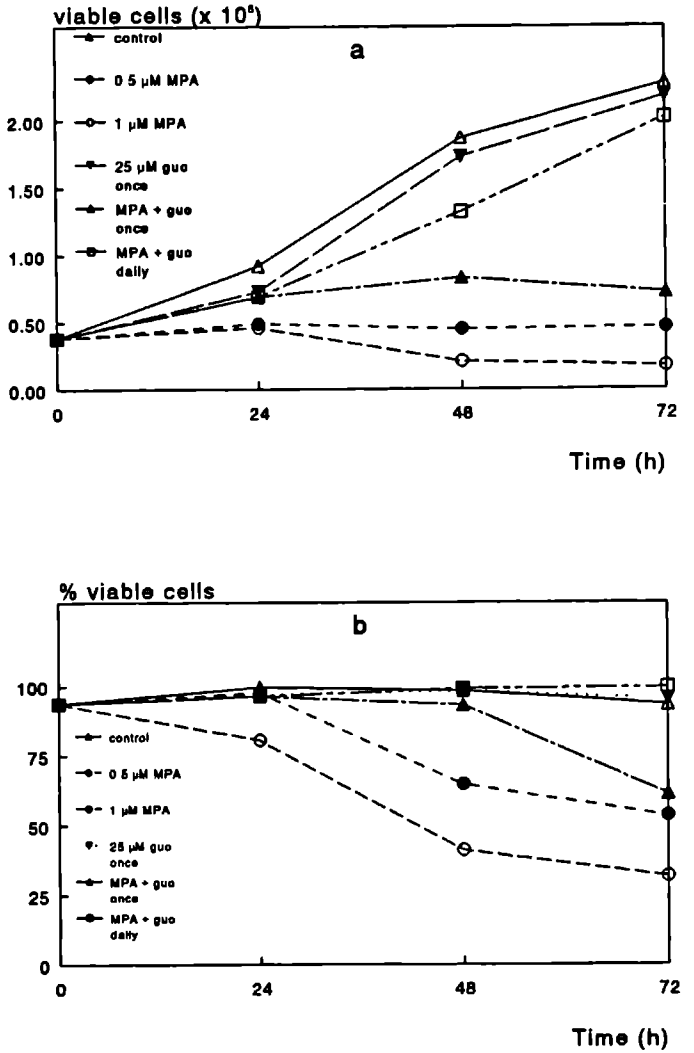

Fig. 1. Cell growth (a) and cell viability (b) of Molt F4 cells after treatment with 0.5 µM MPA, 25 µM guanosine, or a combination of both. Guanosine was added either alone, or in combination with 0.5 µM MPA. Furthermore, effects of daily supplement of guanosine on cell growth and cell viability of untreated and MPA treated cells were assessed. The results of one experiment are shown. Similar results were obtained in two other experiments.

Table I

Purine nucleotide contents of Molt F4 cells treated with 0.5 μM MPA, 25 μM guanosine, or a combination of both, expressed as percentages of untreated cells (median with range of 4 independent experiments). Guanosine was added either once, together with MPA, or every day, to assess the effects of daily supplement of guanosine. Adenine nucleotides (ADP + ATP) of Molt F4 cells before treatment are 4215 (3846-4302) pmoles/10^6 viable cells. Guanine nucleotides (GDP + GTP) before treatment are 843 (766-963) pmoles/10^6 viable cells.

	Adenine Nucleotides				Guanine Nucleotides			
Time (h)	0.5 μM MPA once	25 μM Guo once	MPA + 25 μM Guo, both once	MPA, once + 25 μM Guo, daily	0.5 μM MPA once	25 μM Guo once	MPA + 25 μM Guo, both once	MPA, once + 25 μM Guo, daily
2	97 (97-125)	82 (72-83)	70 (68-84)	82 (72-84)	53 (47-69)	204 (151-218)	202 (141-220)	213 (202-220)
6	108 (93-119)	71 (71-78)	78 (68-81)	71 (71-78)	41 (33-42)	239 (193-320)	256 (215-322)	227 (215-256)
24	97 (90-114)	120 (85-125)	120 (97-138)	124 (120-124)	41 (29-49)	122 (114-241)	34 (29-233)	34 (29-138)
26	96 (78-96)	127 (109-127)	122 (122-137)	105 (105-114)	41 (33-41)	121 (104-121)	34 (34-39)	224 (224-244)
30	84 (84-85)	118 (108-118)	136 (136-138)	93 (93-127)	41 (39-41)	105 (94-105)	31 (31-34)	267 (265-267)
48	88 (83-116)	106 (90-110)	97 (94-113)	121 (115-121)	52 (45-61)	102 (91-105)	52 (44-58)	44 (37-53)

Table II

Pyrimidine nucleotide contents of Molt F4 cells treated with 0.5 μM MPA, 25 μM guanosine, or a combination of both, expressed as percentages of untreated cells (median with range of 4 independent experiments). See further the legend to Table I. Cytosine nucleotides (CDP + CTP) in Molt F4 cells before treatment are 388 (246-539) pmoles/10^6 viable cells; uracil nucleotides (UDP + UTP) before treatment are 1171 (906-1502) pmoles/10^6 viable cells.

	Cytosine Nucleotides				Uracil Nucleotides			
Time (h)	0.5 μM MPA once	25 μM Guo once	MPA + 25 μM Guo, both once	MPA, once + 25 μM Guo, daily	0.5 μM MPA once	25 μM Guo once	MPA + 25 μM Guo, both once	MPA, once + 25 μM Guo, daily
2	110 (104-133)	86 (84-95)	80 (72-90)	87 (80-90)	110 (104-133)	84 (82-86)	81 (75-87)	85 (81-87)
6	121 (100-172)	73 (69-90)	83 (63-107)	83 (69-107)	147 (100-166)	67 (60-79)	78 (50-90)	78 (63-90)
24	99 (74-107)	121 (79-126)	112 (71-154)	131 (112-154)	107 (91-127)	122 (68-125)	109 (63-177)	154 (109-177)
26	83 (66-83)	130 (115-130)	143 (143-157)	102 (102-112)	98 (78-98)	126 (111-126)	162 (162-181)	92 (92-101)
30	65 (65-65)	95 (95-108)	155 (155-156)	95 (95-127)	86 (86-86)	120 (110-120)	190 (190-193)	87 (87-117)
48	97 (77-157)	110 (107-123)	127 (105-176)	165 (153-189)	130 (101-166)	111 (107-123)	162 (137-195)	217 (203-239)

MPA cytotoxicity during the entire incubation period of 72 h (Fig. 1). Addition of guanosine resulted in an increase of intracellular guanine nucleotides and the presence of guanosine and guanine in the medium 2 and 6 h after each addition, as detected at 2, 6, 26 and 30 h (Table I). However, 24 h after addition, guanosine was consumed, since after 48 and 72 h intracellular guanine nucleotide concentrations were depleted again and no guanosine and guanine could be detected in the medium (Tables I and III) prior to the next daily supplement of guanosine.

Table III

Concentrations of nucleosides and bases in culture medium of Molt F4 cells, treated with 0.5 μM MPA alone, or in combination with 25 μM guanosine (see further the legend to Table I). Concentrations are expressed in μmoles/L. Data of one representative experiment are shown (- means : below detection limit).

	Time (h)	CYTIDINE	GUANINE	HYPOXANTHINE	XANTHINE	URIDINE	GUANOSINE
Control	t=0	-	-	0 31	7 12	0 19	-
	t=2	-	-	0 23	7 20	-	-
	t=6	-	-	0 26	6 76	0 33	-
	t=24	-	-	0 15	6 07	0 27	-
	t=48	-	-	0 21	6 14	0 28	-
	t=72	-	-	0 20	5 68	0 33	-
MPA	t=2	-	-	0 37	6 67	0 43	-
	t=6	0 17	-	0 70	6 74	0 87	-
	t=24	0 98	-	0 51	6 24	2 88	-
	t=48	1 61	-	0 59	6 69	4 35	-
	t=72	2 71	-	0 37	6 75	5 34	-
guanosine	t=2	-	13 84	-	6 99	0 14	2 65
	t=6	-	12 98	-	7 89	0 10	0 78
	t=24	-	-	0 15	10 15	0 19	0 09
	t=48	-	-	0 20	9 73	0 40	-
	t=72	-	-	0 16	8 99	-	-
MPA + guanosine	t=2	-	13 76	-	7 07	0 15	2 63
	t=6	-	12 40	-	7 91	0 11	0 82
	t=24	-	-	0 22	9 34	0 75	-
	t=48	0 87	-	0 48	9 23	4 46	-
	t=72	1 20	-	0 80	9 04	5 66	-
guanosine daily	t=26	-	17 38	-	10 37	0 11	2 82
	t=30	-	14 65	-	12 89	0 09	0 90
	t=48	-	-	0 21	12 38	0 53	-
	t=72	-	-	0 51	13 43	0 46	-
MPA + guanosine daily	t=26	-	17 32	-	9 88	0 40	2 39
	t=30	-	13 60	-	11 82	0 07	0 65
	t=48	-	-	0 34	12 51	1 32	-
	t=72	-	-	0 38	15 24	0 11	-

Discussion

In this study IMPDH activity is determined and the effects of its inhibition by MPA are studied in Molt F4 human malignant lymphoblasts.

A direct inhibition of IMPDH by MPA is demonstrated in pre-incubation experiments. However, as a result of pre-incubation, the enzyme activity in the control (without MPA) also decreases. This is possibly due to the absence of the substrate IMP during the pre-incubation period of 20 min., since binding of IMP may exert a stabilizing effect on the enzyme in this in vitro setting. So, from these results it is clear that it is of importance to compare the results of the pre-incubation experiments only with controls which have undergone the same pre-incubation treatment.

0.5 μM MPA induces cytotoxicity (Fig. 1), as a result of depletion of intracellular guanine nucleotides (Table I). Already 6 h after addition of MPA, depletion of guanine nucleotides is maximal. Since the guanine nucleotide pool is much smaller than the adenine nucleotide pool, depletion of guanine nucleotides may rapidly result in inhibition of cell growth and loss of cell viability (22). Inhibition of DNA synthesis after MPA treatment was reported earlier (12,17,21,22).

The rapid increase of the PRPP concentration observed after treatment with 0.5 μM MPA may be explained by feedback-inhibition of PRPP amidotransferase (31) and of hypoxanthine-guanine phosphoribosyltransferase (32) by IMP. The PRPP concentration controls pyrimidine de novo synthesis (33), so the increase of PRPP accounts for the small increase of intracellular cytosine and uracil nucleotides and of extracellular cytosine and uracil nucleosides, observed early after the start of the MPA treatment (Tables II and III). This effect is also observed when the purine de novo synthesis is inhibited by specific inhibitors (13,34-37).

Guanosine was added to the incubations with MPA to determine whether depletion of intracellular guanine nucleotides is the only cause for MPA cytotoxicity in Molt F4 cells. Exogenous guanosine is first converted into guanine by purine nucleoside phosphorylase, and then into GMP by hypoxanthine-guanine phosphoribosyl transferase, an enzyme of the purine salvage route. Guanine is detectable in the medium after addition of guanosine (Table III). Addition of guanosine to incubation with 0.5 μM MPA prevents MPA cytotoxicity (Fig. 1), as described previously (5,12,21,38,39). However, a single addition of 25 μM guanosine to MPA treatment prevents depletion of intracellular guanine nucleotides only during the first 6 h of treatment (Table I). Furthermore, after 24 h no guanine or guanosine are present in the medium (Table III). This indicates a rapid consumption of guanosine by the purine salvage route.

Daily supplements with 25 μM guanosine can maintain cell growth and cell viability at the

level of untreated cells. But still, after 24 h of incubation, prior to the next daily addition of guanosine, intracellular guanine nucleotides are depleted and extracellular guanosine and guanine are undetectable (Tables I and III). So, guanosine consumption by the salvage pathway in these cells is very rapid and 25 μM guanosine is not sufficient to restore intracellular guanine nucleotides during a period of 24 h.

In conclusion, MPA induces cytotoxicity and inhibition of IMPDH activity in Molt F4 human malignant lymphoblasts. Both cytotoxicity and depletion of intracellular guanine nucleotides could be prevented by addition of exogenous guanosine, indicating that depletion of guanine nucleotides is the only mechanism of cytotoxicity of MPA in these cells.

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CHAPTER 4

A BIOCHEMICAL BASIS FOR SYNERGISM OF 6-MERCAPTOPURINE AND MYCOPHENOLIC ACID IN MOLT F4, A HUMAN MALIGNANT T-LYMPHOBLASTIC CELL LINE.

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A biochemical basis for synergism of 6-mercaptopurine and mycophenolic acid in Molt F4, a human malignant T-lymphoblastic cell line

Summary

6-Mercaptopurine (6MP) cytotoxicity was studied in Molt F4 cells, a T-cell acute lymphoblastic leukemia (ALL) cell line. The effects on cytotoxicity were concentration-dependent. Measurements of intracellular thionucleotide intermediates of 6MP demonstrated a rapid rise of thio-IMP (tIMP) levels and subsequently a rapid decrease. Thio-GMP (tGMP) and methyl-thio-IMP (Me-tIMP) appeared later in time, and persisted longer. Mycophenolic acid (MPA), a specific inhibitor of IMP dehydrogenase (IMPDH), was used to inhibit the conversion of tIMP into tGMP, thereby decreasing the incorporation of 6MP into DNA. A synergistic effect on cell viability and cell growth was observed when cells were treated with a combination of 2 μM 6MP and 0.5 μM MPA. Also, intracellular Me-tIMP increased 5 times with the combination. Based on the increase of Me-tIMP concentration and the observed synergism between 6MP and MPA, we conclude that methylation of tIMP into Me-tIMP is an important alternative route for 6MP cytotoxicity.

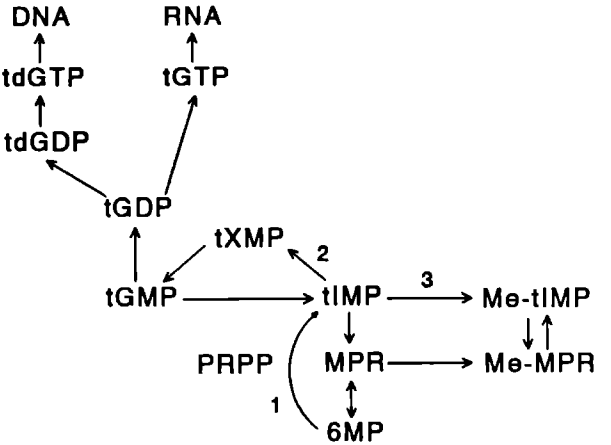
Introduction

6-Mercaptopurine (6MP) is an analogue of the purine base hypoxanthine. It exhibits an antileukemic activity in vitro and in vivo (1) and is commonly used in the maintenance treatment of children with acute lymphoblastic leukemia (ALL), in combination with methotrexate (MTX).

The first step of 6MP metabolism is intracellular uptake and conversion into thio-IMP (tIMP) by the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (2-4). A co-substrate for this reaction is phosphoribosyl pyrophosphate (PRPP). tIMP can be further metabolized by two pathways (see Scheme I).

First, tIMP can be converted into thioguanine nucleotides by enzymes of the purine interconversion pathway (3,5,6). The key enzyme in this route is IMP dehydrogenase (IMPDH) (7,8). Thioguanine nucleotides can be incorporated into RNA and in particulary into DNA (5,9,10). Incorporation of thioguanine nucleotides into DNA results in delayed cytotoxicity by inhibition of transcription of DNA and by induction of DNA damage (11-13), such as single strand breaks and protein cross-links (14,15). Secondly, tIMP can be methylated by the enzyme thiopurine methyltransferase (TPMT) into methyl-tIMP (Me-tIMP) (4,16). Me-tIMP is a strong inhibitor of the first step in the purine de novo synthesis

Scheme I. 6-mercaptopurine metabolism



(1) Hypoxanthine-guanine phosphoribosyl transferase (HGPRT); (2) IMP-dehydrogenase (IMPDH); (3) Thiopurine methyltransferase (TPMT)synthesis (19,20).

(PDNS): PRPP amidotransferase (4,17,18). Inhibition of the PDNS also leads to cytotoxicity, as a result of decreased formation of purine nucleotides for DNA and RNA synthesis (19,20).

Knowledge of the biochemical pathways leading to 6MP cytotoxicity is necessary for understanding of the therapeutic results of 6MP administration in patients with ALL.

In this study, the significance of the methylation route for 6MP cytotoxicity is investigated in Molt F4, a human malignant T-lymphoblastic cell line. For this purpose IMPDH, the rate-limiting enzyme in the purine interconversion route was inhibited by mycophenolic acid (MPA), a specific inhibitor of this enzyme (21-24).

Our studies indicate the importance of Me-tIMP for 6MP cytotoxicity, as demonstrated by a synergistic effect on cell growth and cell viability, with concurrent increase of Me-tIMP and a decreased formation of tGMP after simultaneous administration of 6MP and MPA.

Materials and methods

The experiments were performed with Molt F4 cells, a T-cell acute lymphoblastic leukemia cell line. The cells were cultured in RPMI 1640 DM (Gibco, the Netherlands) supplemented with 10% non-dialyzed fetal calf serum (Gibco, Netherlands), 2 mM L-glutamine (Sigma

Chemicals, USA), 2 mM sodium pyruvate (BDH Chemicals Ltd, UK) and gentamicin/ streptomycin, thrice a week. Cell cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C. The absence of mycoplasma contamination and the presence of marker antigens on the cells were tested regularly.
24 h prior to the start of the experiments, logarithmically growing cells were seeded in a concentration of 0.2 x 10^6 cells per ml. 6MP (Wellcome, Netherlands), MPA (Sigma Chemicals, USA) or a combination of both were added as a single dose in a small volume (1/100). During the experiments 2 mM glutamine was supplemented every 24 h, in order to prevent glutamine exhaustion of the medium (25). After incubation the cells were harvested and counted in a coulter counter. The cell viability was determined by means of trypan blue exclusion. Cell growth was determined by counting the cells and correcting cell number for cell viability.
Endogenous nucleotides were extracted from 3 x 10^6 viable cells by means of perchloric acid (PCA, BDH Chemicals Ltd, UK) extraction. The cells were centrifuged in an Eppendorf centrifuge for 2 min. A volume of 150 μl 0.4 M PCA was added to the cell pellet and the suspension was kept on ice for 10 min. The samples were centrifuged for 2 min. and the supernatant was collected and neutralized with 4 M KH_2PO_4. The samples were stored at -20°C until analysis. The nucleotide concentrations were determined by means of HPLC (26) at a wavelength of 254 nm. The concentrations were expressed as pmoles/10^6 viable cells.
Thionucleotides were extracted from 10^7 viable cells. PCA-extraction was performed with 150 μl 0.4 M PCA and 0.3 % dithiotreitol (DTT, Boehringer, Mannheim) to prevent oxidation of the thio-group. The samples were prepared further as described above and were analyzed by HPLC. Thio-IMP and thio-GMP concentrations were determined at 320 nm and Me-tIMP at 290 nm. The concentrations were expressed as pmoles/10^6 viable cells.

Results

Both 6MP and MPA induced inhibition of cell growth and cytotoxicity (Fig. 1a,b). 6MP cytotoxicity was concentration dependent (data not shown).
When the cells were treated with a combination of 2 μM 6MP and 0.5 μM MPA, a synergistic effect on cell growth and cell viability was observed (Fig. 1a,b). When 10 μM 6MP was used instead of 2 μM, no synergistic effect on cell growth and cell viability could be demonstrated, as a result of the very high cytotoxicity of 10 μM 6MP (data not shown).
The endogenous purine nucleotide pools of cells treated with 2 μM 6MP were reduced during the first 24 h (Table I), while pyrimidine nucleotide concentrations were elevated (data not shown). However, purine nucleotide levels were restored after 48 h. With 10 μM 6MP, depletion of purine nucleotides was more severe and persisted during the entire incubation period of 72 h (Table I).

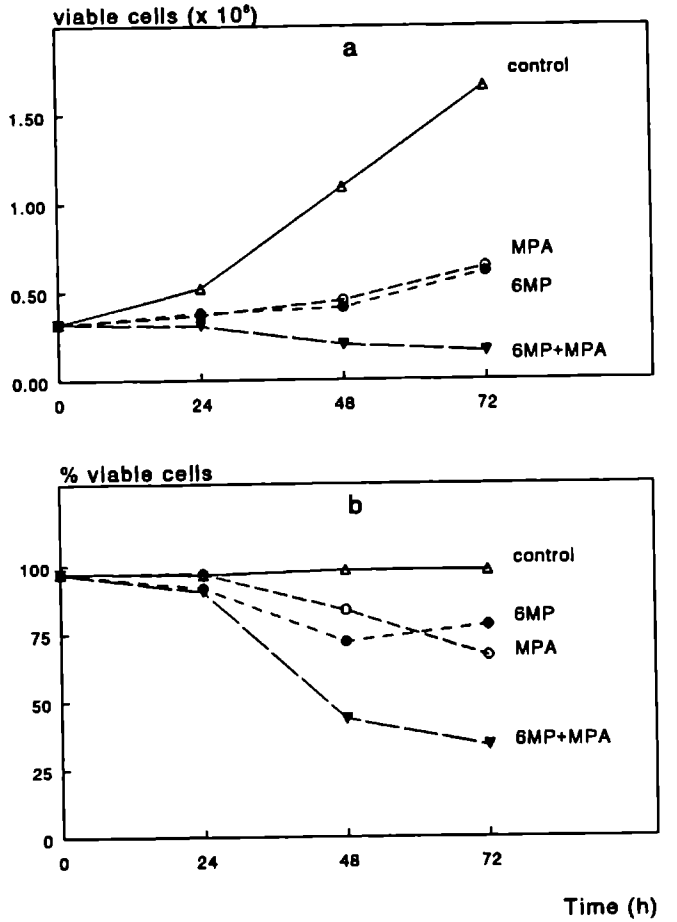


Fig. 1. Cell growth (1a) and cell viability (1b) of Molt F4 cells after treatment with 2 μM 6MP, 0.5 μM MPA or a combination of both. The results one experiment are shown. Similar results were obtained in four other experiments.

When the cells were treated with 0.5 μM MPA the cellular guanine nucleotide pool decreased within 2 h after the start of the incubation and persisted during the entire incubation period of 72 h (Table II). No effects were observed on the adenine nucleotide concentration. The decrease of guanine nucleotides after 6 h with the combination of 2 μM 6MP and 0.5 μM MPA was more severe than with either drug alone (Tables I and II). After 48 h, however, the guanine nucleotide concentration in the cells treated with the combination recovered (Table I), while it remained depressed in cells treated with MPA alone (Table II). The adenine nucleotide concentration remained the same.

Table I: Purine nucleotide contents of Molt F4 cells treated with either 2 or 10 μM 6MP, or a combination of 2 or 10 μM 6MP with 0.5 μM MPA, expressed as percentages of untreated cells (mean with standard error of 5 independent experiments). The purine nucleotide concentrations of Molt F4 cells before treatment are 4750 ± 640 and 890 ± 120 pmoles/10^6 viable cells for adenine and guanine nucleotides, respectively.

	2 μM 6MP				10 μM 6MP			
	ADP + ATP		GDP + GTP		ADP + ATP		GDP + GTP	
MPA	-	+	-	+	-	+	-	+
time (h) 2	99 ± 6	92 ± 19	57 ± 2	40 ± 10	74	86 ± 8	35	34 ± 4
6	82 ± 16	84 ± 9	77 ± 21	38 ± 3	67 ± 10	77 ± 14	32 ± 7	29 ± 4
24	59 ± 16	49 ± 12	73 ± 18	55 ± 6	40 ± 10	40 ± 6	79 ± 25	68 ± 14
48	98 ± 22	80 ± 19	125 ± 19	120 ± 37	63 ± 6	73 ± 13	80 ± 18	60 ± 15
72	102 ± 25	87 ± 51	106 ± 31	74 ± 47	84 ± 5	70 ± 20	78 ± 18	52 ± 21

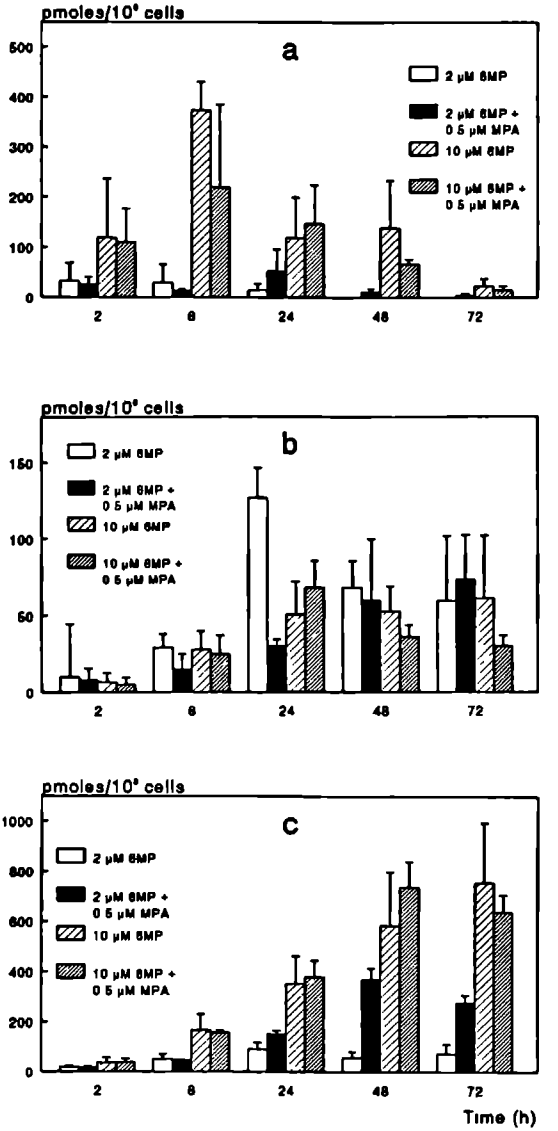


Fig. 2. Thionucleotide contents of Molt F4 cells treated with 2 or 10 µM 6MP either alone or in combination with 0.5 µM MPA (expressed as pmoles/10⁸ viable cells; mean with standard error of 5 independent experiments). (a):tIMP;(b):tGMP;(c):Me-tIMP.

With a combination of 10 μM 6MP and 0.5 μM MPA, nucleotide concentrations did not differ significantly from those observed with 10 μM 6MP alone (Table I). Only the depletion of the guanine nucleotide pool persisted for a longer period of time.
Intracellular formation of the thionucleotides tIMP, tGMP and Me-tIMP could be demonstrated after treatment with 6MP (Fig. 2). With 2 μM 6MP, the tIMP concentration reached its maximum at 2-6 h after the start of the incubation (Fig. 2a), then it declined. tGMP (Fig. 2b) and Me-tIMP (Fig. 2c) concentrations steadily increased to much higher levels during the first 24 h and declined afterwards.
After incubation with 10 μM 6MP, more tIMP was formed, and it remained at a higher level than with 2 μM 6MP (Fig. 2a). The level of Me-tIMP increased continuously during incubation with 10 μM 6MP to a level 10 times higher as compared to the level reached with 2 μM 6MP at 72 h (Fig. 2c). tGMP levels obtained after incubation with 10 μM 6MP were comparable to those obtained after treatment with 2 μM 6MP (Fig. 2b).
Determination of thionucleotide concentrations of cells treated with a combination of 2 μM 6MP and 0.5 μM MPA revealed a prolonged intracellular availability of tIMP as compared to 6MP alone (Fig. 2a). As a result of addition of MPA, the tGMP concentration increased less during the first 24 h as compared to 6MP alone (Fig. 2b). However, no differences could be observed thereafter. The Me-tIMP level was affected most by combination of

Table II: Purine nucleotide contents of Molt F4 cells treated with 0.5 μM MPA, expressed as percentage of untreated cells (mean with standard error of 5 independent experiments). The purine nucleotide concentrations in Molt F4 cells before treatment are 4750 ± 640 and 890 ± 120 pmoles/10^6 viable cells for adenine and guanine nucleotides, respectively.

	ADP + ATP	GDP + GTP
time (h) 2	98 ± 11	48 ± 8
6	104 ± 22	57 ± 15
24	84 ± 19	61 ± 20
48	118 ± 22	80 ± 13
72	85 ± 19	69 ± 17

2 μM 6MP and 0.5 μM MPA (Fig. 2c). It increased considerably as a result of addition of MPA. At 48 h the Me-tIMP concentration with the combination was 5 times as high as compared to 6MP alone.
After combination of 10 μM 6MP and 0.5 μM MPA tIMP and tGMP levels were lower as

compared to 10 μM 6MP alone (Figs. 2a,b). The Me-tIMP concentration with the combination did not differ significantly from that with 10 μM 6MP alone.

Discussion

6MP cytotoxicity can be caused by incorporation of thioguanine nucleotides into DNA and RNA and by formation of Me-tIMP and subsequent inhibition of the PDNS (4,17,18). Molt F4 cells strongly depend on PDNS with regard to formation of purine nucleotides for DNA and RNA synthesis (27). Therefore, inhibition of the PDNS can contribute significantly to the cellular cytotoxicity of 6MP for this malignant lymphoblastic cell line.

Inhibition of the PDNS by Me-tIMP results in accumulation of PRPP. The surplus of PRPP can be used for de novo synthesis of pyrimidine nucleotides (6,19,28,29-31). This is also demonstrated in this study, as uridine and cytidine nucleotides increase to 270 % and 160 % of control, respectively, after a 48-h incubation with 2 μM 6MP (data not shown).

In cells treated with 2 μM 6MP formation of thionucleotides as well as Me-tIMP can be demonstrated (Fig. 2). The long persistence of Me-tIMP levels, associated with depletion of its precursor 6MP in the medium 6 h after the start of the treatment (32), indicates a long half-life of this metabolite (33). After treatment with 10 μM 6MP the tIMP concentration is over 10 times as high as compared to 2 μM 6MP at 6 h. In spite of this, tGMP concentrations with 10 μM 6MP do not exceed those of cells treated with 2 μM 6MP. This may be caused by two phenomena. First, high concentrations of tIMP cause inhibition of IMPDH (4), and thus formation of tGMP. Secondly, because ATP is a cofactor for the enzyme GMP-synthetase, the severe depletion of the adenine nucleotide pool with 10 μM 6MP may lead to inhibition of this enzyme and thus a decreased formation of tGMP. Therefore, it is necessary to reassess the effects of low and high concentrations of 6MP on cell growth and cell viability. With 10 μM 6MP a tremendous amount of Me-tIMP is formed as compared to 2 μM 6MP (Fig. 2) and relatively less 6MP is incorporated into DNA, especially after 24 h (32). Inhibition of the PDNS by Me-tIMP leads to a nearly complete cessation of RNA and DNA synthesis, especially after 48 h (32). Also, cytotoxicity with 10 μM 6MP becomes apparent early after the start of the treatment, whereas 2 μM 6MP hardly affects cell viability at that time. These observations lead to the conclusion that during treatment with high concentrations of 6MP, the methylation route is the most important mechanism for 6MP cytotoxicity, whereas with lower concentrations of 6MP incorporation into DNA leads to delayed cytotoxicity after 48 h of incubation (5).

Inhibition of IMPDH by MPA is especially cytotoxic for tumor cells (30). IMPDH was shown to have a higher activity in tumor cells as compared to normal cells (34), probably as a response to the high purine nucleotide need of tumor cells, necessary for DNA and RNA synthesis. In Molt F4 cells depletion of guanine nucleotides is already observed 2 h after addition of MPA (Table II). These results are similar with those obtained in other studies

(35-37).

The synergistic effects on cell growth and cell viability (Fig. 1) by the combination of 2 μM 6MP and 0.5 μM MPA clearly confirm the importance of the methylation route for 6MP cytotoxicity. As a result of the inhibition of IMPDH by MPA, more tIMP is available for methylation into Me-tIMP. Moreover, the increase of Me-tIMP concentration resulting from combination of both drugs leads to enhanced inhibition of PDNS, causing a more severe depletion of purine nucleotides, especially of guanine nucleotides (Table I).

The results of our studies are in contrast with the studies of Lennard et al. (38,39), who claim that methylation of tIMP into Me-tIMP is a detoxification pathway for 6MP cytotoxicity. In patients as a whole this may well be true, but with respect to the cellular level Me-tIMP may have an additional effect on 6MP cytotoxicity. Their studies were performed in red blood cells, which lack an active PDNS and DNA synthesis, in contrast to malignant lymphoblasts. Because malignant lymphoblasts are the ultimate target for 6MP cytotoxicity in the treatment of ALL, Molt F4 human malignant lymphoblasts are a better model system to study the biochemical route for 6MP cytotoxicity. Another reason for underestimating the role of Me-tIMP for 6MP cytotoxicity may be that in patients the bioavailability of orally administered 6MP may be too low (40,41) to result in adequate Me-tIMP levels in red blood cells and peripheral lymphocytes.

In conclusion, the methylation pathway of 6MP is important for cytotoxicity in Molt F4 malignant human lymphoblasts. Synergism with respect to cytotoxicity is observed under conditions where an elevation of Me-tIMP concentration is measured and incorporation of 6MP into DNA is at least partly blocked by MPA.

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CHAPTER 5

THE IMPORTANCE OF METHYLTHIO-IMP FOR METHYLMERCAPTOPURINE RIBONUCLEOSIDE (ME-MPR) CYTOTOXICITY IN MOLT F4 HUMAN MALIGNANT T-LYMPHOBLASTS.

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The importance of methylthio-IMP for methylmercaptopurine ribonucleoside (Me-MPR) cytotoxicity in Molt F4 human malignant T-lymphoblasts

Summary

The importance of methyl-thioIMP (Me-tIMP) formation for methylmercaptopurine ribonucleoside (Me-MPR) cytotoxicity was studied in Molt F4 cells. Cytotoxicity of Me-MPR is caused by Me-tIMP formation with concomitant inhibition of purine de novo synthesis. Inhibition of purine de novo synthesis resulted in decreased purine nucleotide levels and enhanced 5-phosphoribosyl-1-pyrophosphate (PRPP) levels, with concurrent increased pyrimidine nucleotide levels. The Me-tIMP concentration increased proportionally with the concentration of Me-MPR. High Me-tIMP concentration also caused inhibition of PRPP synthesis. Maximal accumulation of PRPP thus occurred at low Me-MPR concentrations. As little as 0.2 μM Me-MPR resulted already after 2 h in maximal inhibition of formation of adenine and guanine nucleotides, caused by inhibition of purine de novo synthesis by Me-tIMP. Under these circumstances increased intracellular PRPP concentrations could be demonstrated, resulting in increased levels of pyrimidine nucleotides. So, in Molt F4 cells, formation of Me-tIMP from Me-MPR results in cytotoxicity by inhibition of purine de novo synthesis.

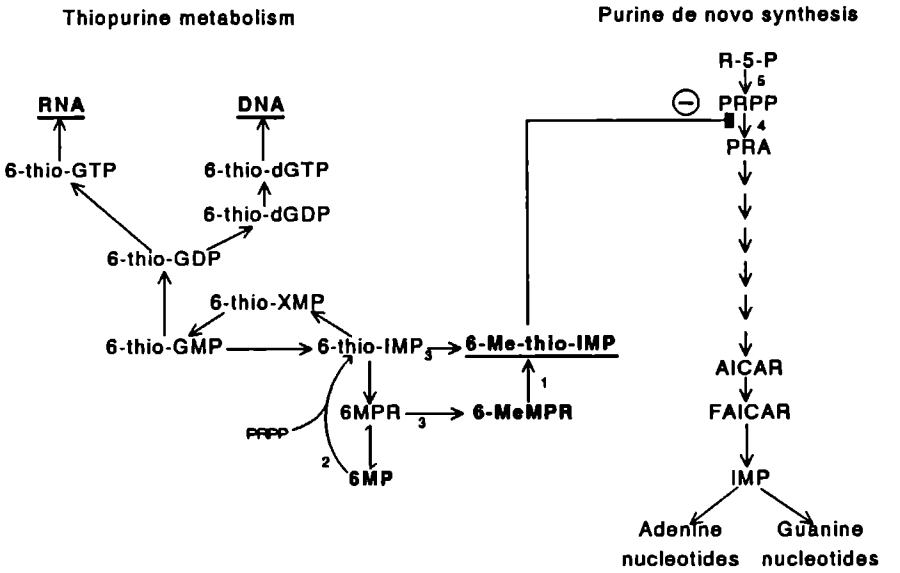
Introduction

Methyl-thioIMP (Me-tIMP) is a strong inhibitor of purine de novo synthesis at the level of the enzyme phosphoribosylpyrophosphate amidotransferase (scheme 1) (1-3), causing cytotoxicity and especially inhibition of cell growth, as a result of decreased formation of purine nucleotides for DNA and RNA synthesis (4-7).

The cytotoxicity of methylmercaptopurine ribonucleoside (Me-MPR) is caused by its conversion into Me-tIMP (5,8,9). Me-MPR can be converted into Me-tIMP by the enzyme adenosine kinase with ATP as co-substrate (8,10-12) (scheme 1). Me-MPR is a potent inhibitor of the growth of several mouse tumors and of human tumor cells grown in culture (13,14). Incubation of a variety of tumor cell lines with Me-MPR leads to decreased purine de novo synthesis (5,15,16), resulting in decreased purine nucleotide levels, increased concentrations of 5-phosphoribosyl-1-pyrophosphate (PRPP) (15,17,18) and enhanced pyrimidine nucleotide levels (4,16).

Me-tIMP formation is also important for cytotoxicity of 6-mercaptopurine (6MP), an analogue of hypoxanthine (7). 6MP is converted into thio-IMP (tIMP) by the enzyme hypoxanthine-guanine phosphoribosyltransferase with PRPP as co-substrate (1,19,20). Further

Scheme 1. Purine metabolism.



(1) Adenosine kinase (AK); (2) Hypoxanthine-guanine phosphoribosyl transferase (HGPRT); (3) Thiopurine methyltransferase (TPMT); (4) Phosphoribosylpyrophosphate amidotransferase; (5) Phosphoribosylpyrophosphate synthetase (PRPPs).

conversion of tIMP occurs by two routes. Firstly, t-IMP can be converted into thioguanine nucleotides (20-22). Subsequently, these thioguanine nucleotides can be incorporated into RNA or DNA (6,21,23,24) which results in delayed cytotoxicity (21,25). Secondly, tIMP can be methylated by thiopurine methyltransferase into Me-tIMP, which inhibits purine de novo synthesis (1,26). Both metabolic routes of 6MP are important for cytotoxicity (7). 6MP exhibits an antileukemic activity in vitro and in vivo (27) and is commonly used in the maintenance treatment of children with acute lymphoblastic leukemia in combination with methotrexate.

In this study, the effects of Me-tIMP on cell growth and cell viability, PRPP levels and purine and pyrimidine nucleotide concentrations are investigated in Molt F4 cells, a human T-lymphoblastic cell line, by treating the cells with various concentrations of Me-MPR or 6MP.

Materials and methods

6MP was purchased from Wellcome, the Netherlands; Me-MPR, PRPP (sodium salt), the yeast orotidine-5-phosphate ribosyltransferase/orotidine-5-phosphate decarboxylase were obtained from Sigma Chemicals, USA. [^{14}C-Carboxyl] orotic acid (52.5 mCi/mmol) was obtained from NEN Products, MA, USA.

The studies were performed with Molt F4 cells, a T-cell acute lymphoblastic leukemia cell line. All experiments were started with an initial concentration of 0.2 x 10^6 cells/ml 24 h before addition of the drugs. Me-MPR and 6MP were added as a single dose in a small volume (1/100). Conditions for cell culture and experimental procedures have been described earlier (7).

Determination of endogenous nucleotides and Me-tIMP:

Endogenous nucleotides (di- and triphosphates) and Me-tIMP were determined by means of HPLC (7,28). The nucleotide concentrations were determined at 254 nm and Me-tIMP at 290 nm. Concentrations were expressed as pmoles/10^6 viable cells.

PRPP assay:

We used the assay for determination of PRPP as previously described (29-32), but with a few modifications. The PRPP concentration was determined by its enzymatic conversion with [^{14}C-carboxyl] orotic acid to form $^{14}CO_2$ and uridine monophosphate.

A 1.0 ml aliquot of cell suspension was centrifuged in an Eppendorf centrifuge for 2 min. The cell pellet was suspended in 250 μl 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. After vortexing, the cells were lysed by sonification (Branson sonifier, 4 bursts of 10 sec at 20% of maximal output) and vortexed again. 100 μl suspension (in duplicate) was transferred in a glass vial which was sealed with a rubber cap.

Enzymes in this suspension were inactivated by heating for 45 sec in boiling water. After the heating step, the reaction was initiated by adding 10 μl orotidine-5-phosphate ribosyltransferase/orotidine-5-phosphate decarboxylase (7.7 mg/ml) in 25 mM $MgCl_2$ solution, followed by 5 μl 1.17 mM [^{14}C-carboxyl] orotic acid (17 mCi/mmol). Immediately afterwards, a second smaller vial, containing 0.7 ml hyamine, was inserted in the reaction vial and the vial was sealed again instantly. While shaking, the reaction was performed for 90 min at 37°C and immediately stopped afterwards with 50 μl of 4 N $HClO_4$. Shaking was continued for 90 min to trap all $^{14}CO_2$ in the inner vial. The vial with hyamine was transferred to another glass vial containing 5 ml of Toluene Scintillator and the amount of trapped $^{14}CO_2$ was counted in a liquid scintillation counter.

Some PRPP was destroyed during the heating step. The mean recovery of standard PRPP solutions added to cell lysates was 88.0% (S.D. = 3.8, N = 11). So, a correction was

made for the percentage of PRPP lost during heating. The assay was linear between 50 pmoles and 3000 pmoles (corr. coeff. : 0.9997, N = 7).

Results

When Molt F4 cells were incubated with various concentrations of Me-MPR, a concentration dependent inhibition of both cell growth and cell viability was observed (Figs. 1a,b). From 0.1 to 0.5 μM Me-MPR, cell growth and cell viability decreased with increasing concentration of Me-MPR. With 0.5 μM Me-MPR, cell growth exhibited a minimum of 15 % of the control and cell viability about 60% of the control at 48 h of incubation.
Maximal conversion of Me-MPR into Me-tIMP took place within the first 2 h (Table 1).

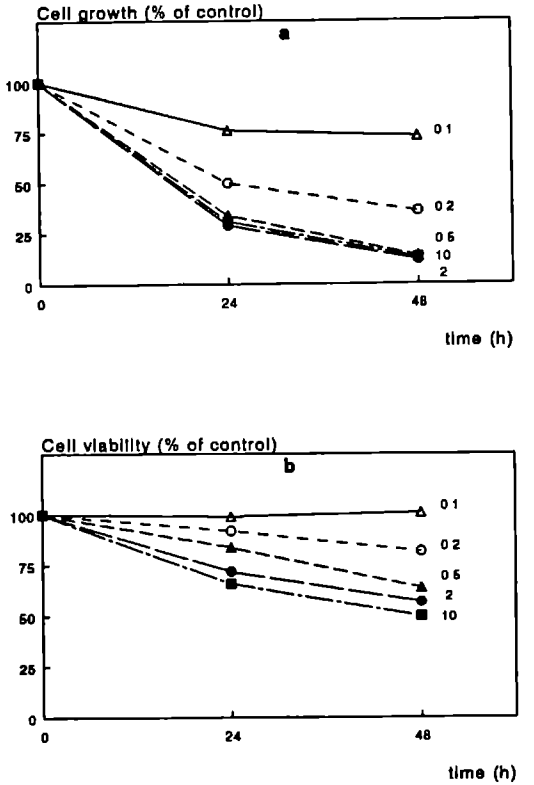


Fig. 1. Cell growth (a) and cell viability (b) of Molt F4 cells after treatment with various concentrations Me-MPR (expressed as percentages of untreated cells; the results of one experiment are shown. Similar results were obtained in two other experiments).

Table 1. Me-tIMP levels in Molt-F4 cells treated with various concentrations of Me-MPR (expressed as pmoles/10^6 viable cells; median with range (between brackets) of 3 independent experiments).

time (h)	0.1 μM Me-MPR	0.2 μM Me-MPR	0.5 μM Me-MPR	2.0 μM Me-MPR	10 μM Me-MPR
2	281 (275-359)	560 (514-596)	1130 (1127-1234)	3264 (2810-3377)	3922 (3583-4469)
6	271 (254-291)	520 (484-527)	1123 (1088-1706)	2431 (2381-2753)	4007 (3661-4007)
24	145 (117-148)	437 (386-450)	1170 (1019-1170)	3406 (3332-4035)	4623 (3434-5161)
48	40 (39-42)	225 (223-225)	1276 (1165-1358)	3634 (2902-3667)	4506 (3504-4644)

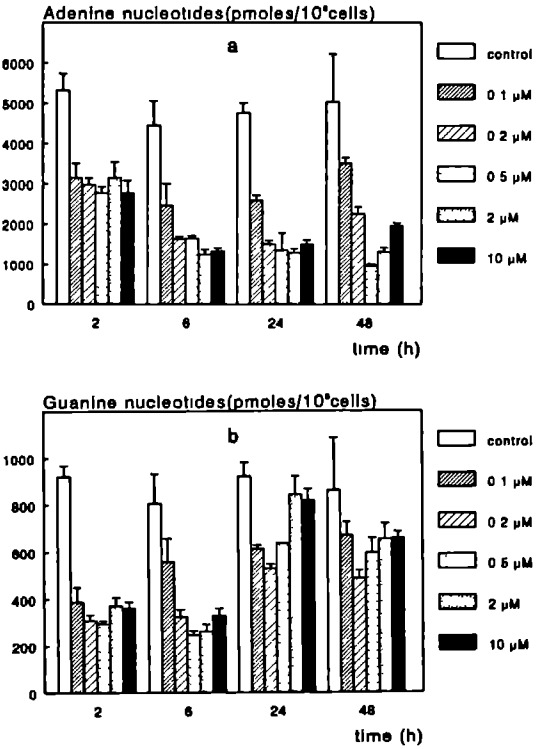


Fig. 2. Adenine (a) and guanine (b) nucleotides (di- and triphosphates) of Molt F4 cells treated with various concentrations Me-MPR (expressed as pmoles/10^6 cells; mean with standard error of 3 independent experiments).

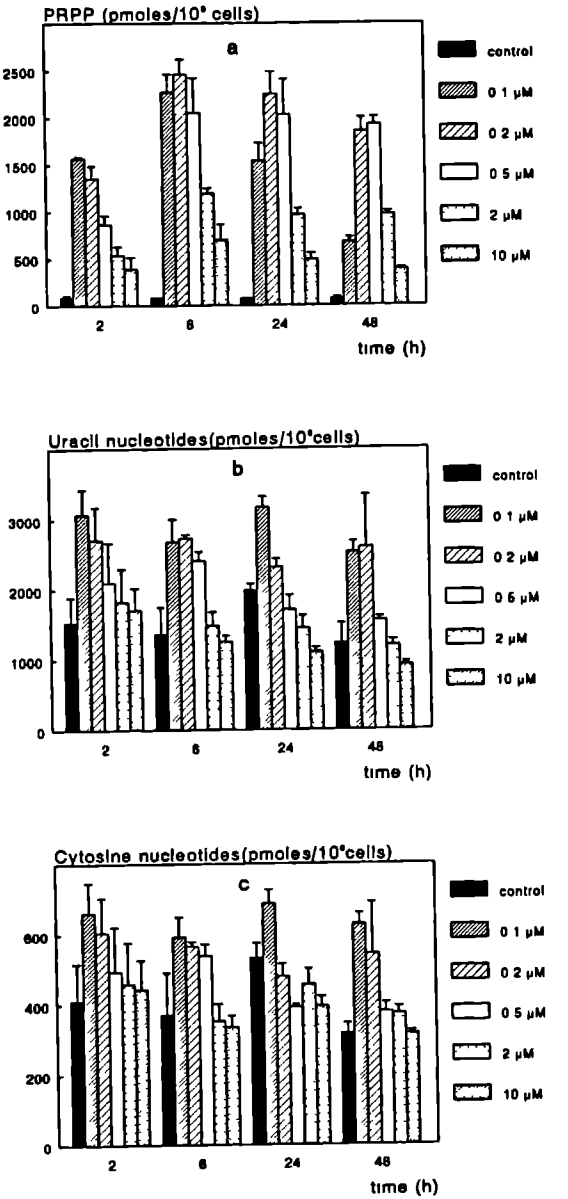


Fig. 3. PRPP levels (a) and uracil (b) and cytosine (c) nucleotides in Molt F4 cells treated with various concentrations Me-MPR (expressed as pmoles/10⁶ cells; mean with standard error of 3 independent experiments).

The Me-tIMP concentration increased with increasing Me-MPR concentrations. However, with 10 μM Me-MPR a slightly higher increment was observed compared with 2 μM Me-MPR after 2 h. Me-tIMP concentrations obtained with 0.1 μM and 0.2 μM Me-MPR reached a maximum at 2 h and decreased again after 6 h. With higher concentrations of Me-MPR the Me-tIMP concentrations remained at a high level during the whole incubation period.

Adenine and guanine nucleotide concentrations decreased after treatment with Me-MPR (Figs. 2a,b). A minimal adenine nucleotide concentration of 30% of the control was already achieved with 0.2 μM Me-MPR after 6 h and no difference was observed with increasing concentrations of Me-MPR. Recovery of the adenine nucleotide concentration of cells, treated with 0.1 and 0.2 μM Me-MPR, appeared after 48 h. Guanine nucleotides exhibited a minimum of about 35% of control after 2 h, while the recovery appeared within 24 h.

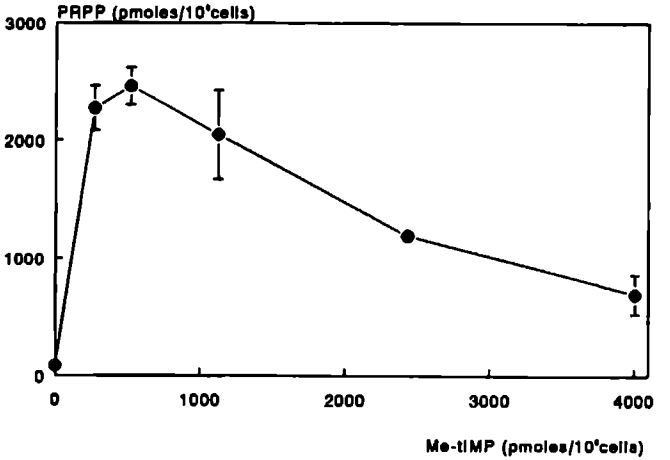


Fig. 4. PRPP levels as a function of Me-tIMP levels after 6 h of incubation with various concentrations of Me-MPR, derived from Table 1 and Fig. 3a. (mean of 3 independent experiments; Me-tIMP concentrations were expressed as nmoles/10⁶ cells; PRPP concentrations were expressed as nmoles/10⁶ cells).

Fig. 3a shows the effects of various concentrations of Me-MPR on intracellular PRPP levels in Molt F4 cells. PRPP levels rapidly increased after incubation with Me-MPR, especially at low concentrations of Me-MPR. Maximal accumulation of PRPP occurred at 6 h, with a 25-fold increase of PRPP level after incubation with 0.2 μM Me-MPR. After an initial increase of PRPP levels after incubation with 0.1 μM Me-MPR and to a lesser extent with 0.2 μM Me-MPR, the PRPP concentration decreased after 6 h, whereas PRPP levels obtai-

ned with higher Me-MPR concentrations hardly changed. In general, the changes in PRPP levels closely resembled that of pyrimidine nucleotide levels. Endogenous pyrimidine nucleotides increased with 0.1 to 0.5 μM Me-MPR (Figs. 3b,c). Maximal increments after 2 h appeared with 0.1 μM Me-MPR. At this time uracil nucleotides increased to 200% and cytosine nucleotides to 160% of the control. With 0.1 μM and 0.2 μM Me-MPR the elevated pyrimidine nucleotide levels persisted during the entire incubation period of 48 h, whereas the levels after incubation with 0.5 μM Me-MPR recovered to control values after 48 h of incubation.

Table 2. PRPP levels in Molt F4 cells treated with various concentrations 6MP (expressed as pmoles/10^6 cells; mean with standard error of 4 independent experiments). PRPP levels in untreated cells were: 108 ± 28 pmoles/10^6 cells.

time (h)	2 μM 6MP	10 μM 6MP
4	441 ± 56	454 ± 78
8	623 ± 113	527 ± 50
24	789	126
48	1080	146

Fig. 4 demonstrates the correlation between PRPP accumulation and Me-tIMP levels, measured after 6 h of incubation with various concentrations of Me-MPR (Table 1 and Fig. 3a). After an initial increase of intracellular PRPP concentration to a maximum at 500 pmoles Me-tIMP/10^6 viable cells, PRPP accumulation decreased with higher Me-tIMP concentrations.

Me-tIMP formation is also an important step for 6MP cytotoxicity (7,33). The effects of 6MP on PRPP levels are demonstrated in table 2. With 2 μM 6MP a 6-fold increase in PRPP level within 8 h was followed by a slower increase during the remaining incubation period. Treatment of cells with 10 μM 6MP caused an initial 5-fold increment of PRPP levels within 8 h, followed by a decrease after 24 h to control values.

Discussion

In order to study the influence of Me-tIMP on cell growth, cell viability, nucleotide pools and PRPP levels, Molt F4 cells were incubated with various concentrations of Me-MPR.

The effect of Me-MPR on cell growth was more severe than on cell viability (Figs. 1a,b).

Decreased cell growth and unaltered cell viability after treatment with 0.1 μM Me-MPR indicates that cell growth depends on an intact purine de novo synthesis in rapidly dividing malignant cells (34).

The direct conversion of Me-MPR into Me-tIMP was demonstrated in Table 1, showing an almost maximal Me-tIMP formation for all Me-MPR concentrations at 2 h of incubation (16). The relatively small increment of Me-tIMP from 2 μM to 10 μM Me-MPR may be a consequence of depletion of ATP (Fig. 2a). As ATP is a co-substrate for the conversion of Me-MPR into Me-tIMP by adenosine kinase, depletion of ATP may lead to suboptimal activity of this enzyme.

The decrease of Me-tIMP concentration with 0.1 μM Me-MPR after 24 and 48 h (Table 1) was accompanied by a decrease of PRPP levels (Fig. 3a), indicating that Me-tIMP indeed accounts for the increased PRPP levels. The lesser extent of PRPP accumulation with higher Me-MPR concentrations (Fig. 3a), associated with higher Me-tIMP concentrations (Table 1), might be explained by an inhibitory effect of Me-MPR on the synthesis of PRPP. This could also be demonstrated in Fig. 5, showing a decrease of PRPP levels with increasing intracellular Me-tIMP levels above 500 pmol/10^6 cells. Yen et al. also described inhibition of PRPP synthetase with high concentrations of Me-MPR by the Me-MPR metabolite, Me-tIMP, in human fibroblasts (35). Moreover, decrease of PRPP synthetase activity with increased Me-MPR concentrations could also be caused by the decreased availability of ATP, the co-substrate for PRPP synthetase. Firstly, ATP is consumed for the conversion of Me-MPR into Me-tIMP by adenosine kinase. Secondly, the inhibition of the purine de novo synthesis by Me-tIMP results in a concurrent decrease of ATP formation (Fig. 2a). So, with high concentrations of Me-MPR more ATP will be consumed and ATP formation will be inhibited more severely, resulting in less availability of ATP for PRPP synthesis.

The depletion of adenine and guanine nucleotides (Figs. 2a,b) reflects the effect of Me-MPR on purine de novo synthesis (16). The more persistent decrease of adenine nucleotides compared to guanine nucleotides is probably due to competitive inhibition of adenosine kinase by Me-MPR. As a result of this, conversion of adenosine into AMP and subsequently into ATP cannot occur optimally. In addition, purine precursors, e.g. from dead cells, become available for guanine nucleotide synthesis by the salvage pathway and purine interconversion.

Accumulation of PRPP, especially at low Me-MPR concentrations, caused an increase of pyrimidine nucleotide levels within 6 h of incubation with Me-MPR (Figs. 3b,c). This observation is in agreement with earlier observations (11),(12). The results of both purine nucleotides and PRPP levels, indicate that a maximal inhibition of the purine de novo synthesis is already achieved with 0.2 μM Me-MPR, corresponding with 500 pmoles Me-tIMP/10^6 viable cells. Under these conditions PRPP levels are maximal and endogenous purine nucleotide levels are minimal (Fig. 4).

Incubation of cells with 6MP, another anticancer drug of which Me-tIMP is an active metabolite, also led to increased PRPP levels (Table 2). Me-tIMP, formed after methylation of thio-IMP by thiopurine methyltransferase, is responsible for this elevation of PRPP levels. Previous research showed that with 6MP maximal Me-tIMP concentration occurred after 24 h (7). This is in contrast with already maximal levels of Me-tIMP after 2 h of incubation with Me-MPR (Table 1). These data may indicate that the methylation enzyme thiopurine methyltransferase has to be induced, to result in maximal Me-tIMP concentrations at later times after incubation with 6MP, as has been described in literature (36,37). The increase of PRPP levels with 2 μM 6MP up to 48 h confirm this phenomenon (Table 2).

The decline of PRPP levels to control values after prolonged incubation with 10 μM 6MP, despite of the increase of Me-tIMP concentration (7), seems to be in contrast. However, under these conditions reutilization of nucleotide precursors, that become available from dead cells as a consequence of the high cytotoxicity of 10 μM 6MP, occurs. This is associated with PRPP consumption. Moreover, inhibition of PRPP synthetase by high concentrations of Me-tIMP may result in the observed decrease of the PRPP levels to control values.

In conclusion, Me-tIMP is a potent inducer of inhibition of both cell growth and cell viability in Molt F4 cells, which exhibit a highly active purine de novo synthesis. Therefore, Me-tIMP is an important metabolite for Me-MPR and 6MP cytotoxicity in cells with an active purine de novo synthesis.

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CHAPTER 6

REVERSAL OF 6-MERCAPTOPURINE AND 6-METHYLMERCAPTOPURINE RIBONUCLEOSIDE CYTOTOXICITY BY AMIDOIMIDAZOLE CARBOXAMIDE RIBONUCLEOSIDE IN MOLT F4 HUMAN MALIGNANT T-LYMPHOBLASTS.

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Reversal of 6-mercaptopurine and 6- methylmercaptopurine ribonucleoside cytotoxicity by amidoimidazole carboxamide ribonucleoside in Molt F4 human malignant T-lymphoblasts

Summary

Cytotoxicity of 6-mercaptopurine (6MP) and 6-methylmercaptopurine ribonucleoside (Me-MPR) was studied in Molt F4 human malignant lymphoblasts. Both drugs are converted into methyl-thioIMP (Me-tIMP), which inhibits purine de novo synthesis. Addition of amidoimidazole carboxamide ribonucleoside (AICAR) circumvented inhibition of purine de novo synthesis, and thus partly prevented 6MP and Me-MPR cytotoxicity. Purine nucleotides, and especially adenine nucleotides recovered by addition of AICAR. Under these conditions, Me-tIMP formation decreased. The results of this study indicate that formation of Me-tIMP may be important for 6MP cytotoxicity in Molt F4 cells. These data suggest that depletion of adenine nucleotides is the main cause for Me-tIMP cytotoxicity.

Introduction

6-Mercaptopurine (6MP), an analogue of the purine base hypoxanthine, is used in the maintenance treatment of children with acute lymphoblastic leukemia. The first step in 6MP cytotoxicity is conversion into thioIMP (tIMP) (1,2). tIMP can be metabolized by two pathways. First, it is converted into thioguanine nucleotides, which can be incorporated into the RNA and DNA of the cells, leading to DNA-damage and delayed cytotoxicity (3,4). Second, it is methylated into methyl-thioIMP (Me-tIMP) by the enzyme thiopurine methyltransferase (EC 2.1.1.67) (1,5). Since Me-tIMP is a strong inhibitor of purine de novo synthesis (6-8) it induces cytotoxicity by depletion of purine ribonucleotides.

In the present study the importance of Me-tIMP for 6MP cytotoxicity was examined in more detail. Amidoimidazole carboxamide ribonucleoside (AICAR), an intermediate of purine de novo synthesis distal to the site where Me-tIMP exerts its inhibiting effect, was used to circumvent inhibition of this route by Me-tIMP. AICAR is phosphorylated into AICAR monophosphate by adenosine kinase (AK, EC 2.7.1.20) (9,10) and can enter purine de novo synthesis in this form. Me-tIMP cytotoxicity was examined more specifically using 6-methylmercaptopurine ribonucleoside (Me-MPR). This is also converted by AK (11) into Me-tIMP. Again AICAR was used to prevent inhibition of purine de novo synthesis.

Materials and Methods

Mycophenolic acid (MPA), Me-MPR and AICAR were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); 6MP from Wellcome (Utrecht, The Netherlands). The experiments were performed with Molt F4, a human T-cell acute lymphoblastic leukemia cell line. Conditions for cell culture and experimental procedures have been described in detail previously (12).

Endogenous nucleotides (di- and triphosphates) were determined in 3 x 10^6 viable cells, according to the method described earlier (13), and were measured at a wavelength of 254 nm. Nucleotide concentrations were expressed as pmoles/10^6 viable cells. Thionucleotides were extracted from 10^7 viable cells, according to the procedure described earlier (12). tIMP and tGMP were determined at 320 nm. Me-tIMP was measured at 290 nm. Concentrations were expressed as pmoles/10^6 viable cells.

Results

Treatment of Molt F4 cells with 2 μM 6MP induced cytotoxicity (Table 1) and led to intracellular depletion of purine nucleotides (Table 2). Depletion of guanine nucleotides occurred more rapidly than that of adenine nucleotides, but recovered earlier. Complete recovery of the guanine nucleotides could be observed after 48 hr. Maximal depletion of the adenine nucleotide pool occurred after 24 hr and lasted longer. Simultaneous addition of AICAR to treatment with 2 μM 6MP partly prevented cytotoxicity and resulted in a decre-

Table I. Cell number and cell viability of Molt F4 cells after 48 hr exposure to 2 μM 6MP, 0.5 μM MPA or a combination of both, and to 0.5 μM Me-MPR with or without addition of 50 μM AICAR. Cell number is expressed as 10^5 viable cells/ml. Cell viability is expressed in %; mean ± SD of three independent experiments. ND: not done.

	Cell number		Cell viability	
	AICAR		AICAR	
	-	+	-	+
control	1.64 ± 0.15	1.71 ± 0.24	97.5 ± 0.8	97.4 ± 1.0
2 μM 6MP	0.65 ± 0.25	0.98 ± 0.15	78.0 ± 12.6	90.9 ± 4.4
0.5 μM MPA	0.70 ± 0.1	ND	75.7 ± 2.6	ND
2 μM 6MP + 0.5 μM MPA	0.27 ± 0.09	0.57 ± 0.06	47.4 ± 6.5	68.0 ± 1.8
0.5 μM Me-MPR	0.27 ± 0.06	0.79 ± 0.12	60.6 ± 1.6	91.0 ± 3.1

ased depletion of adenine nucleotides as compared to 6MP alone (Table 2). Depletion of guanine nucleotides could be partly prevented by AICAR during the first 2 hr. Decreased formation of tIMP and tGMP (results not shown) and of Me-tIMP (Fig. 1) was observed in cells treated with 6MP and AICAR as compared to 6MP alone. When 2 μM 6MP was combined with 0.5 μM MPA, an inhibitor of the conversion of tIMP into thioguanine nucleotides under these conditions, more Me-tIMP was formed and cytotoxicity was potentiated (12). Addition of 50 μM AICAR to this treatment resulted in cytotoxicity comparable to that of MPA alone (Table 1).

Inhibition of purine de novo synthesis by Me-tIMP was examined further using Me-MPR as its precursor. Me-MPR (0.5 μM) induced inhibition of cell growth and cell viability (Table 1) and resulted in depletion of purine nucleotides (Table 2). Addition of 50 μM AICAR to this treatment led to a complete recovery of cell viability and a partial recovery of cell growth (Table 1). Under these conditions adenine nucleotide concentrations partly recovered (Table 2), while guanine nucleotides recovered only during the first 6 hr of the treatment. Moreover, the intracellular formation of Me-tIMP was reduced as a result of treatment of cells with a combination of 0.5 μM Me-MPR and AICAR as compared to 0.5 μM Me-MPR alone, especially after prolonged incubation (Fig. 1).

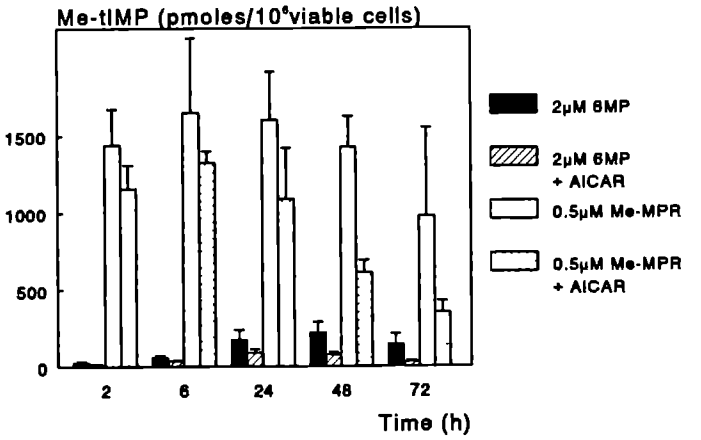

Fig. 1. Me-tIMP concentration of Molt F4 cells treated with 2 μM 6MP or 0.5 μM Me-MPR alone, or in combination with 50 μM AICAR (expressed as pmoles/10⁶ viable cells; mean with standard error of 3 independent experiments).

Table II

Purine nucleotide concentrations of Molt F4 cells treated with 2 μM 6MP or 0.5 μM Me-MPR alone, or in combination with 50 μM AICAR Values are expressed as % of untreated cells; median and range (between brackets) of three experiments.

The purine nucleotide concentrations of untreated Molt F4 cells are 5502 ± 495 and 921 ± 61 pmoles/10^6 viable cells for adenine and guanine nucleotides, respectively.

time (h)	Adenine nucleotides				Guanine nucleotides			
	6MP	6MP + AICAR	Me-MPR	Me-MPR + AICAR	6MP	6MP + AICAR	Me-MPR	Me-MPR + AICAR
2	83 (42-93)	89 (88-109)	83 (65-89)	84 (71-100)	66 (22-66)	79 (50-83)	68 (41-82)	76 (61-93)
6	78 (73-135)	83 (77-102)	50 (28-80)	69 (67-85)	52 (39-93)	60 (56-69)	56 (53-94)	80 (63-81)
24	52 (40-58)	70 (61-86)	43 (33-49)	60 (52-95)	75 (40-79)	65 (50-86)	88 (79-89)	64 (60-110)
48	54 (53-82)	88 (82-166)	40 (35-46)	55 (34-55)	102 (83-103)	98 (54-236)	78 (71-78)	71 (45-72)
72	83 (72-104)	106 (101-112)	30 (18-37)	61 (50-80)	101 (99-128)	110 (109-122)	57 (30-67)	68 (59-70)

Discussion

In the present study the contribution of Me-tIMP formation to 6MP cytotoxicity is examined in Molt F4 cells. 6MP cytotoxicity is partly prevented by addition of AICAR to 6MP treatment (Table 1). Furthermore, AICAR ameliorates 6MP cytotoxicity in experiments where MPA is used to inhibit 6MP conversion into thioguanine nucleotides.

Earlier research showed that with a combination of 2 μM 6MP and 0.5 μM MPA more Me-tIMP is formed, and 6MP cytotoxicity is potentiated (12). Addition of AICAR to treatment with 0.5 μM MPA and 2 μM 6MP reduces cytotoxicity to a level obtained with MPA alone (Table 1).

Prevention of 6MP cytotoxicity by AICAR may be attributed to two mechanisms. First, as a result of addition of AICAR the effects of inhibition of purine de novo synthesis by Me-tIMP can be circumvented. This is demonstrated by the partial recovery of the adenine nucleotides (Table 2) after addition of AICAR. Second, Me-tIMP formation is decreased as a result of addition of AICAR to 6MP treatment (Fig. 1). This may be a consequence of phosphoribosylpyrophosphate consumption by the intracellular metabolism of AICAR (14) and may result in the availability of less phosphoribosylpyrophosphate for 6MP conversion into tIMP, and subsequently less intracellular thioguanine nucleotide formation. This complicates elucidation of the effects of addition of AICAR on 6MP cytotoxicity. Therefore, combinations of Me-MPR and AICAR were used to investigate the effect of AICAR on inhibition of purine de novo synthesis by Me-tIMP more thoroughly, as Me-MPR is converted directly into Me-tIMP by the enzyme AK (11). This conversion is reflected by rapid formation of very high concentrations of Me-tIMP in the cells treated with 0.5 μM Me-MPR (Fig. 2). The more severe depletion of adenine nucleotides as compared to guanine nucleotides by 0.5 μM Me-MPR (Table 2) could be ascribed both to consumption of ATP by AK, during conversion of Me-MPR into Me-tIMP, and to inhibition of adenine nucleotide formation as a consequence of competitive inhibition of AK by Me-MPR. Addition of AICAR restored cell viability and growth of cells treated with Me-MPR (Table 1). This is in accordance with data reported on amidoimidazolecarboxamide (AIC), which is also converted into AICAR monophosphate and is able to reverse cytotoxicity of Me-MPR (15). Depletion of adenine nucleotides was partly prevented by addition of AICAR to Me-MPR treatment (Table 2). Simultaneously, a remarkable decrease in Me-tIMP concentration was observed (Fig. 1), probably as a result of competition for AK between AICAR and Me-MPR. However, since 500 pmol Me-tIMP/10^6 viable cells induce complete inhibition of the purine de novo synthesis (16), the partial recovery of the adenine nucleotides observed here still is a result of circumvention of inhibition of purine de novo synthesis by AICAR, rather than of a suboptimal inhibition of purine de novo synthesis by a decreased Me-tIMP concentration.

The partial recovery of adenine nucleotides by addition of AICAR (Table 2) was associated

with an almost complete recovery of cell viability and less inhibition of cell growth (Table 1). Therefore, depletion of adenine nucleotides by Me-tIMP must be the main cause of Me-tIMP cytotoxicity in Molt F4 cells.

In conclusion, 6MP and Me-MPR cytotoxicity can be prevented by AICAR. These data confirm that formation of Me-tIMP may be a second important route for 6MP cytotoxicity. Whether the methylation route of tIMP is important for the therapeutic effects of 6MP in patients remains to be elucidated. The bioavailability of orally administered 6MP is low and under these conditions Me-tIMP formation may not be significant enough to inhibit purine de novo synthesis (12). Under these conditions the methylation route may even function as a detoxification pathway, as described by Lennard et al (17,18). However, when 6MP is administered intravenously very high levels are reached (19) and the methylation route may indeed contribute to 6MP cytotoxicity (12).

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CHAPTER 7

REVERSAL OF METHYLMERCAPTOPURINE RIBONUCLEOSIDE CYTOTOXICITY BY INTERMEDIATES OF PURINE SALVAGE PATHWAY.

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Reversal of methylmercaptopurine ribonucleoside cytotoxicity by intermediates of purine salvage pathway

Summary

Methyl-thioIMP (Me-tIMP), the sole metabolite of Me-MPR is a strong inhibitor of purine de novo synthesis, inducing depletion of intracellular purine nucleotides and subsequent cell death in several tumor cell lines. In this study prevention of methylmercaptopurine ribonucleoside (Me-MPR) cytotoxicity by intermediates of the purine salvage pathway was studied in Molt F4 human malignant T-lymphoblasts. Adenosine, adenine and inosine were able to prevent depletion of the adenine nucleotide pool when used in combination with 0.5 μM Me-MPR, but had virtually no effect on depletion of guanine nucleotides. Nevertheless, these three intermediates of purine salvage were able to ameliorate the cytotoxic effects induced by Me-MPR. Addition of guanosine to treatment with 0.5 μM Me-MPR repleted the guanine nucleotide pool, but adenine nucleotides remained depleted. Under these conditions, inhibition of cell growth was slightly ameliorated. With a combination of guanosine and 10 μM Me-MPR cytotoxicity was increased as compared with 10 μM Me-MPR alone, associated with a depletion of adenine nucleotides to 9 % of untreated cells. Since cell growth and cell viability of Molt F4 cells are restored under conditions where adenine nucleotide depletion is reversed by intermediates of the purine salvage pathway and where the other nucleotides are depleted, we conclude that depletion of adenine nucleotides is an important factor for Me-MPR cytotoxicity.

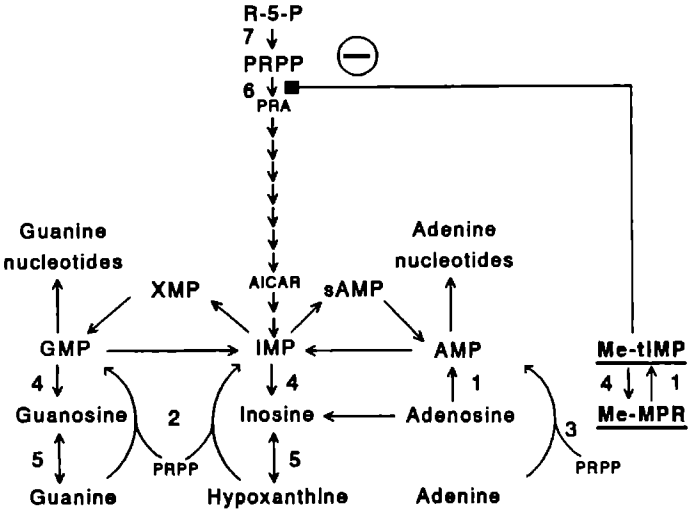
Introduction

Methylmercaptopurine ribonucleoside (Me-MPR), a thiopurine antimetabolite, is cytotoxic for a number of cell lines and has some anticancer activity in vivo (1-5). Cytotoxicity of Me-MPR is mediated by its metabolite methyl-thioIMP (Me-tIMP), which is formed from Me-MPR by adenosine kinase (Scheme I) (3,6-8). Me-tIMP is a strong inhibitor of the purine de novo synthesis (9,10) at phosphoribosylpyrophosphate (PRPP) amidotransferase (9,11-13). Inhibition of this route induces a depletion of purine nucleotides (4,5,14-16), thereby leading to diminution of RNA and DNA formation (14) and subsequent inhibition of cell growth and loss of cell viability (3-5,15).

Me-tIMP is also an important metabolite of the anticancer agent 6-mercaptopurine (6MP). 6MP is first converted into thioIMP (tIMP) and the latter into Me-tIMP by thiopurine methyltransferase (17-19). 6MP is commonly used in the oral maintenance treatment of children with acute lymphoblastic leukemia (17,18).

At present it is under discussion whether formation of Me-tIMP contributes to the anti-

Scheme I. Purine salvage pathway



(1) Adenosine kinase (AK); (2) Hypoxanthine guanine phosphoribosyltransferase (HGPRT); (3) Adenine phosphoribosyltransferase (APRT); (4) 5' Nucleotidase (5'NT); (5) Purine nucleoside phosphorylase (PNP); (6) Phosphoribosylpyrophosphate (PRPP) amidotransferase; (7) phosphoribosylpyrophosphate (PRPP) synthetase.

cancer activity of orally administered 6MP (19). The metabolic route by which 6MP is generally thought to induce cytotoxicity is conversion into 6-thioguanine nucleotides and subsequent incorporation into DNA and RNA (19,20). Furthermore, a high activity of thiopurine methyltransferase in red blood cells, resulting in high Me-tIMP concentrations, is correlated with a poor prognosis in children receiving oral 6MP-therapy, suggesting that the methylation route of 6MP is a catabolic pathway (17,18). Our studies in Molt F4 cells, a human malignant lymphoblastic cell line, indicated that under conditions where intracellular Me-tIMP concentrations were elevated cytotoxicity of 6MP was increased (21). Cytotoxicity of both 6MP and Me-MPR could be reversed in these cells by addition of amidoimidazolecarboxamide ribonucleoside, an intermediate of purine de novo synthesis distal to the Me-tIMP inhibition site (22), further suggesting the cytotoxic potency of Me-tIMP in these cells.

In the present study we obtained more evidence regarding cytotoxicity of Me-tIMP in Molt F4 human T-lymphoblasts. Cell growth, cell viability, endogenous nucleotide concentra-

tions, extracellular nucleosides and bases and formation of Me-tIMP were determined in experiments where cells were treated with various concentrations of Me-MPR alone, or in combination with adenosine, adenine, inosine and guanosine, in order to determine whether cytotoxicity of Me-MPR could be prevented by purine intermediates of the purine salvage route (Scheme I).

Materials and methods

Me-MPR, adenosine, adenine, inosine and guanosine were obtained from Sigma Chemicals, USA. The experiments were performed with Molt F4 cells, a T-cell acute lymphoblastic leukemia cell line. Conditions for cell culture and experimental procedures have been described earlier (21). Me-MPR and adenosine, adenine, inosine, guanosine or combinations Me-MPR with one of these purine salvage intermediates were added as a single dose in a small volume (1/100).

Intracellular nucleotides (di- and triphosphates) and Me-tIMP were extracted from 3 x 10^6 viable cells by means of perchloric acid (PCA, BDH Chemicals Ltd, UK) extraction as previously described (21) and analyzed by means of HPLC at a wavelength of 254 nm (23). The concentrations were expressed as pmoles/10^6 viable cells.

Extracellular nucleosides and bases were extracted from 0.5 ml of the medium, to which a volume of 25 μl 8 M PCA was added. This suspension was kept on ice for 10 min. Then the samples were centrifuged for 2 min., after which the supernatant was neutralized with 4 M K_2HPO_4. Nucleosides and bases were determined by means of reversed-phase HPLC, with a Supelcosil LC-18-DB column (25 cm x 4.6 mm, Supelco, USA), and were detected at a wavelength of 254 nm. Concentrations were expressed as μmoles/l.

Results

Treatment of Molt F4 cells with 0.5 μM Me-MPR resulted in decreased purine nucleotide concentrations (Table I) and led to inhibition of cell growth and of cell viability (Fig. 1). The effects of 10 μM Me-MPR on these parameters were similar (results not shown).

Addition of 50 μM adenosine or adenine or 25 μM inosine to treatment with 0.5 μM Me-MPR resulted in repletion of the intracellular adenine nucleotide pool within the first 24 h of the treatment (Table I). Adenosine was also able to restore the guanine nucleotide pool after 24 h. Adenine and inosine hardly affected the depletion of guanine nucleotides (Table I). These purine salvage intermediates were able to restore cell growth partially and cell viability nearly completely (Fig. 1). Combination of these purine salvage intermediates with 10 μM Me-MPR led to similar results as with 0.5 μM Me-MPR, except for the reversal of depletion of guanine nucleotides by adenosine (results not shown). Addition of 25 μM guanosine to Me-MPR treatment resulted in an increase of intracellular guanine nucleotides, but had little effect on the depletion of adenine nucleotides (Table I). Furthermore,

Table I. Adenine and guanine nucleotide concentrations (di- and triphosphates) of Molt F4 cells treated with 0.5 μM Me-MPR alone, or in combination with 50 μM adenosine or adenine or 25 μM inosine or guanosine. Data are expressed as percentages of untreated cells; median and range (between brackets) of 3 experiments. The adenine nucleotide concentration of Molt F4 cells before treatment is 5180 ± 509 pmoles/10^8 viable cells. The guanine nucleotide concentration of Molt F4 cells before treatment is 963 ± 59 pmoles/10^8 viable cells.

	adenine nucleotides					guanine nucleotides				
time (h)	Me-MPR	Me-MPR + Ado	Me-MPR + Ade	Me-MPR + Ino	Me-MPR + Guo	Me-MPR	Me-MPR + Ado	Me-MPR + Ade	Me-MPR + Ino	Me-MPR + Guo
2	79 (65-93)	111 (95-126)	90 (81-97)	108 (103-129)	54 (43-56)	63 (57-70)	71 (65-73)	57 (51-67)	80 (75-88)	121 (86-159)
6	53 (44-62)	87 (74-88)	99 (78-102)	99 (80-100)	46 (41-49)	59 (47-60)	62 (61-63)	64 (57-66)	70 (61-75)	290 (257-342)
24	47 (36-49)	136 (123-157)	133 (128-147)	97 (91-107)	60 (77-55)	74 (60-83)	98 (80-109)	72 (70-91)	69 (62-73)	403 (392-484)
48	49 (35-55)	47 (40-50)	48 (45-52)	34 (29-35)	69 (49-110)	65 (59-75)	58 (55-70)	65 (55-68)	62 (55-64)	73 (73-123)
72	38 (27-41)	32 (30-37)	31 (30-33)	27 (25-38)	40 (30-47)	64 (48-75)	69 (56-84)	69 (67-76)	70 (63-81)	52 (50-72)

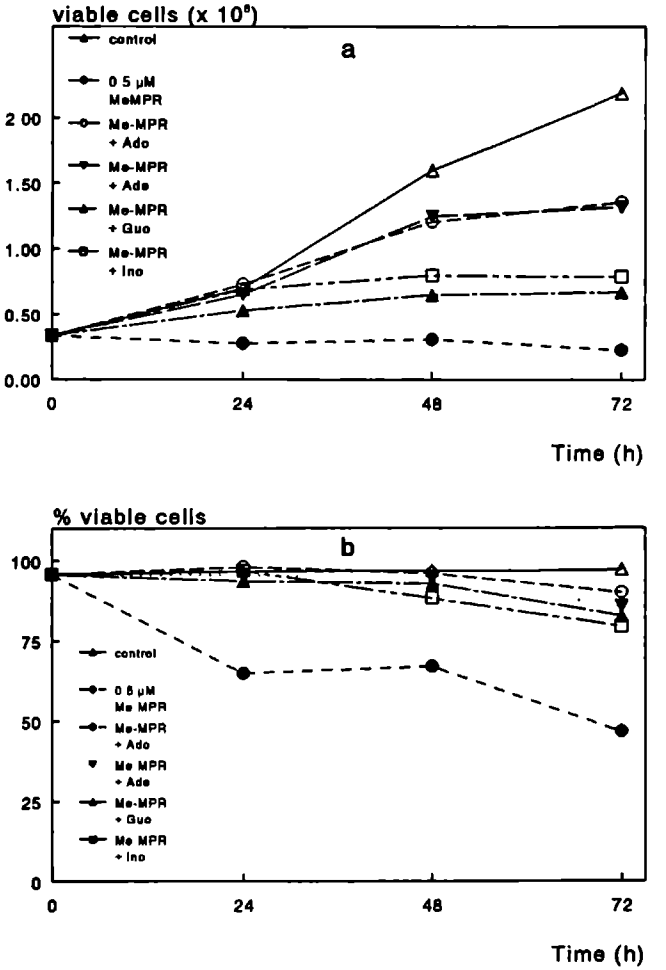


Fig. 1. Cell growth (a) and cell viability (b) of Molt F4 cells after treatment with 0.5 µM Me-MPR alone or in combination with 50 µM adenosine, 50 µM adenine or 25 µM inosine or 25 µM guanosine. The results of one experiment are shown. Similar results were obtained in two other experiments.

this combination resulted in an almost complete recovery of cell viability (Fig. 1b). Cell growth also partially restored (Fig. 1a). In contrast, guanosine in combination with 10 µM Me-MPR led to a large increase of cytotoxicity as compared to 10 µM Me-MPR alone. Under these conditions intracellular adenine nucleotides were depleted to 9 % of untreated

cells after 24 h (results not shown).

To obtain insight in the metabolic fate of the purine intermediates after addition to the cells alone, or in combination with Me-MPR, concentrations of extracellular nucleosides and bases were determined. The results of these experiments are shown in tables II and III.

Adenosine can be incorporated via 2 pathways; directly by adenosine kinase and indirectly by conversion into inosine, which is then converted into hypoxanthine and subsequently into IMP (Scheme I). This second route was reflected by the presence of extracellular ino-sine and hypoxanthine after addition of 50 μM adenosine to Molt F4 cells (Table II). No extracellular adenosine was detected. When inosine is used as intermediate, inosine is first catabolized into hypoxanthine, which is subsequently converted by hypoxanthine-guanine phosphoribosyltransferase into IMP. Both inosine and hypoxanthine were detectable in the medium the first 6 h after the start of the experiment (Table II). Adenine is metabolized directly by adenine phosphoribosyltransferase into AMP and is present in the medium until 48 h (Table III). After addition of guanosine alone, both guanosine and guanine are present (Table III). In general the disappearance of the purine salvage intermediates from the medium was slower when added in combination with 10 μM Me-MPR.

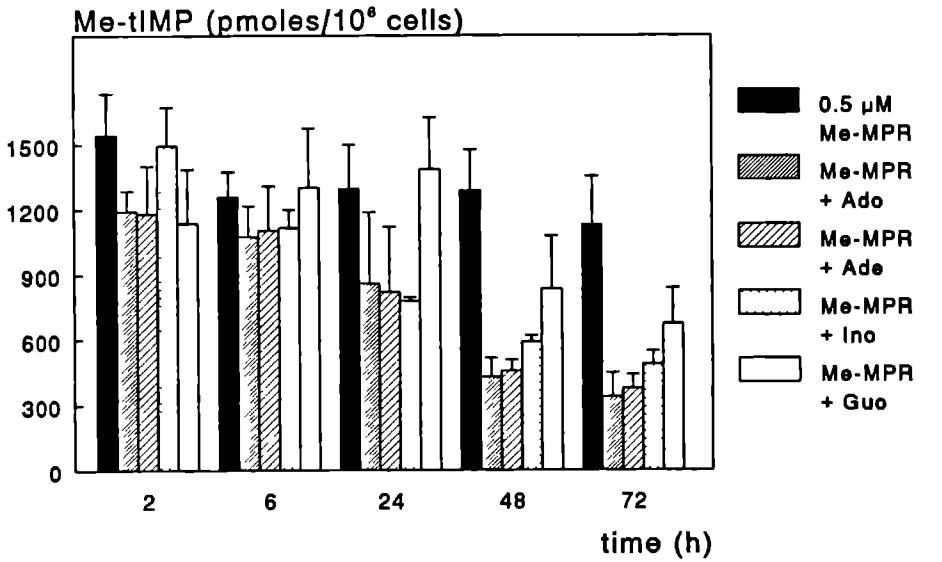


Fig. 2. Me-tIMP concentrations of Molt F4 cells treated with 0.5 μM Me-MPR alone or in combination with the purine salvage intermediates described in Fig. 1 (expressed as pmoles/10⁶ viable cells; mean with standard error of 3 independent experiments).

Table II

Medium concentrations of hypoxanthine, xanthine and inosine of experiments in which Molt F4 cells were treated with 0.5 or 10 μM Me-MPR alone, or in combination with 50 μM adenosine or 25 μM inosine. Concentrations are expressed in μmoles/l. Data of one representative experiment are shown (- means : below detection limit).

	time (h)	control	0.5 μM Me-MPR	10 μM Me-MPR	50 μM adenosine	0.5 μM Me-MPR + 50 μM adenosine	10 μM Me-MPR + 50 μM adenosine	25 μM inosine	0.5 μM Me-MPR + 25 μM inosine	10 μM Me-MPR + 25 μM inosine
Hypoxanthine	2	0.3	0.3	0.3	23.8	22.1	25.2	13.9	13.3	16.1
	6	0.3	0.3	0.5	39.0	35.5	35.9	18.1	18.6	20.2
	24	0.4	0.5	0.5	11.9	15.4	25.1	0.6	0.5	8.7
	48	0.5	0.5	0.6	0.4	0.5	13.1	0.4	0.5	0.5
	72	0.4	0.4	0.5	0.4	0 4	0.4	0.4	0.4	0.5
Xanthine	2	7.8	7.2	7.5	13.5	6.3	7.0	6.4	6.2	7.2
	6	8.7	8.5	8.5	8.1	8.1	7.5	8.3	8.0	8.1
	24	7.7	7.6	7.4	6.7	7.6	7.5	7.3	7.4	7.3
	48	7.5	8.1	8.9	7.9	8.3	9.4	7.9	8.1	8.0
	72	7.0	7.9	8.9	7 5	7.3	8.2	7.4	7.6	7.8
inosine	2	-	-	-	12.3	10.7	12.3	3.1	3.0	4.4
	6	-	-	-	2.3	2.2	2.6	0.7	0.5	0.8
	24	-	-	-	0.2	-	-	-	-	-
	48	-	-	-	-	-	-	-	-	-
	72	-	-	-	-	-	-	-	-	-

Table III

Medium concentrations of adenine, guanosine and guanine of experiments in which Molt F4 cells were treated with 0.5 or 10 μM Me-MPR alone, or in combination with 50 μM adenine or 25 μM guanosine. Concentrations are expressed in μmoles/l. Data of one representative experiment are shown (- means : below detection limit).

	time (h)	control	0.5 μM Me-MPR	10 μM Me-MPR	50 μM adenine	0.5 μM Me-MPR + 50 μM adenine	10 μM Me-MPR + 50 μM adenine	25 μM guanosine	0.5 μM Me-MPR + 25 μM guanosine	10 μM Me-MPR + 25 μM guanosine
Adenine	2	-	-	-	38.5	42.5	30.62	-	-	-
	6	-	-	-	32.1	35.8	35.9	-	-	-
	24	-	-	-	11.3	14.7	21.1	-	-	-
	48	-	-	-	0.3	-	9.6	-	-	-
	72	-	-	-	-	-	-	-	-	-
Guanosine	2	-	-	-	-	-	-	5.4	3.5	5.0
	6	-	-	-	-	-	-	2.5	1.4	2.0
	24	-	-	-	-	-	-	-	-	0.4
	48	-	-	-	-	-	-	-	-	0.5
	72	-	-	-	-	-	-	-	-	-
Guanine	2	-	-	-	-	-	-	16.5	13.9	18.0
	6	-	-	-	-	-	-	20.1	21.2	21.9
	24	-	-	-	-	-	-	-	6.7	18.5
	48	-	-	-	-	-	-	-	-	23.1
	72	-	-	-	-	-	-	-	-	18.2

Me-tIMP concentrations were determined to evaluate the effects of the purine salvage intermediates on Me-MPR metabolism. Combination of 0.5 μM Me-MPR with the purine salvage intermediates resulted in a decrease of the Me-tIMP concentrations after 48 h (Fig. 2). With 10 μM Me-MPR, addition of adenosine, adenine and inosine resulted in a decrease of Me-tIMP concentration. However, addition of guanosine led to a higher Me-tIMP concentration after 48 h as compared to 10 μM Me-MPR alone (5663 $\pm$ 482 and 4063 $\pm$ 351 pmoles/10^6 viable cells, respectively).

Discussion

Me-MPR, an anticancer drug, exerts its cytotoxic activity by conversion into Me-tIMP, a strong inhibitor of the purine de novo synthesis (9,10). In this study the effects of Me-MPR on Molt F4 human T-lymphoblasts are determined.

Inhibition of cell growth, cell viability and purine nucleotide concentrations (Fig. 1, Table I) is already maximal with 0.5 μM Me-MPR (24). With 10 μM Me-MPR no additional effects on cytotoxicity and on purine nucleotides are observed. Because Molt F4 cells have a highly active purine de novo synthesis (25), these cells are highly susceptible for Me-tIMP cytotoxicity.

Adenosine, adenine and inosine are able to ameliorate Me-MPR-induced inhibition of purine de novo synthesis, leading to a partial restoration of cell growth and a nearly complete restoration of cell viability (Fig. 1, Table I). The effects of these three intermediates of purine salvage on purine nucleotide concentrations are comparable. The initial repletion during the first 24 h of intracellular adenine nucleotides is followed by depletion (Table I). This is the result of the very rapid conversion of the purine salvage intermediates, as determined by the rapid disappearance of these intermediates and their derivates from the incubation medium (Tables II and III). Reversal of the effects of 3 μM Me-MPR on induction of differentiation, cell growth inhibition and purine nucleotide concentrations by various concentrations of adenine was observed earlier in HL-60 cells (15) and in sarcoma 180 cells (26).

With 10 μM Me-MPR restoration of the intracellular adenine nucleotide pool as a result of addition of the purine salvage intermediates is less pronounced (results not shown). This can be attributed to two phenomena. First, when adenosine is used in combination with a high concentration of Me-MPR, competition for adenosine kinase may occur, since both adenosine and Me-MPR are metabolized by this enzyme (Scheme I). As a result, less adenosine can be metabolized by the cells. This process is reflected by the prolonged presence of extracellular hypoxanthine (Table II), which is derived from adenosine catabolism and which indicates a slow anabolism of adenosine under these conditions. Second, it is known from the literature (27,28) and from our earlier research (24) that a high Me-tIMP concen-

tration results in inhibition of PRPP synthetase. As PRPP is a cofactor for the enzymes adenine phosphoribosyltransferase and hypoxanthine guanine phosphoribosyltransferase and thus is involved in the metabolism of adenine and inosine (the latter being first converted into hypoxanthine), less adenine and inosine will be incorporated into the cells with 10 μM Me-MPR. Again, this is reflected by the concentrations of extracellular adenine and hypoxanthine after treatment with 10 μM Me-MPR in combination with either inosine (Table II) or adenine (Table III).
The minor effects of adenine, adenosine and inosine on the intracellular guanine nucleotide depletion (Table I) are probably the result of the importance of the adenine-guanine nucleotide balance, which results in the selective restoration of the adenine nucleotide concentration. Moreover, interconversion into guanine nucleotides is a much slower process than interconversion into adenine nucleotides (29).

The severe cytotoxicity observed with the combination of 10 μM Me-MPR and 25 μM guanosine can be ascribed to a nearly complete depletion of the intracellular adenine nucleotides, which is induced by several mechanisms. First, inhibition of the purine de novo synthesis by Me-MPR will result in a depletion of the adenine nucleotides. Second, Me-MPR is converted into Me-tIMP by the enzyme adenosine kinase, a reaction which consumes ATP and thus induces a further decrease of adenine nucleotide concentrations. Third, addition of guanosine leads to an increase of guanine nucleotides of 2.5 times the control value at 48 h (results not shown). The formation of GDP and GTP from GMP consumes ATP by kinase reactions. Therefore the adenine nucleotide pool will be depleted further, leading to the dramatic reduction of intracellular adenine nucleotides observed in these experiments. Combination of guanosine with 0.5 μM Me-MPR does not induce such a severe depletion of the adenine nucleotides (Table I), since at this concentration of Me-MPR less ATP is consumed as a consequence of the adenosine kinase-mediated conversion of Me-MPR into Me-tIMP. Exacerbation of cytotoxicity of Me-MPR by guanosine was reported earlier (15,30,31) and was explained by these authors by a synergistic action between GMP and Me-tIMP, resulting in a more severe inhibition of purine de novo synthesis, presumably at PRPP amidotransferase (30). The results from our study do not confirm this conclusion, since the guanine nucleotide pools are also elevated with the combination of 0.5 μM Me-MPR and guanosine, which does not lead to a more severe depletion of adenine nucleotides as compared to 0.5 μM Me-MPR alone (Table I). The more severe inhibition of the purine de novo synthesis with the combination of 10 μM Me-MPR and guanosine rather is the result of the severe depletion of the adenine nucleotide pool, observed under these conditions. For one, the conversion of ribose-5'-phosphate to PRPP is ATP dependent, so severe depletion of ATP may lead to less availability of PRPP for purine de novo synthesis (24).
The decrease of Me-tIMP concentrations as a result of addition of adenosine as compared to

treatment with Me-MPR alone (Fig. 2) may be the result of competition between adenosine and Me-MPR for adenosine kinase, since both compounds are metabolized by this enzyme. It is at present not clear how addition of adenine, inosine and guanosine affects Me-tIMP formation.

In conclusion, depletion of the intracellular adenine nucleotide concentration appears an important factor for Me-MPR cytotoxicity. This is in contrast with earlier observations that the biological consequences of purine starvation as a result of Me-MPR treatment were primarily a consequence of guanine nucleotide depletion in a mouse T-lymphoma cell line (14). As a result of addition of adenosine, adenine and inosine to treatment with Me-MPR, the adenine nucleotides restored to control values within the first 24 h of the treatment (Table I), whereas guanine nucleotides remained depleted (Table I) and pyrimidine nucleotides (data not shown) became depleted as a result of higher consumption of PRPP by the purine salvage intermediates. Since under these conditions cell growth and cell viability of Molt F4 cells still partly recovered to control values, restoration of the depletion of adenine nucleotides is also important for amelioration of the effects of inhibition of purine de novo synthesis by Me-MPR. Furthermore, it appears of importance to use at least two concentrations of Me-MPR in stu-dying the effects of purine salvage intermediates on Me-MPR induced cytotoxicity, since this reveals more of the underlying mechanisms of cytotoxicity.

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CHAPTER 8

REDUCTION OF S-ADENOSYLMETHIONINE SYNTHESIS BY 6-MERCAPTOPURINE AND METHYLMERCAPTOPURINE RIBONUCLEOSIDE IN MOLT F4 HUMAN MALIGNANT LYMPHOBLASTS.

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Reduction of S-adenosylmethionine synthesis by 6-mercaptopurine and methylmercaptopurine ribonucleoside in Molt F4 human malignant lymphoblasts

Summary

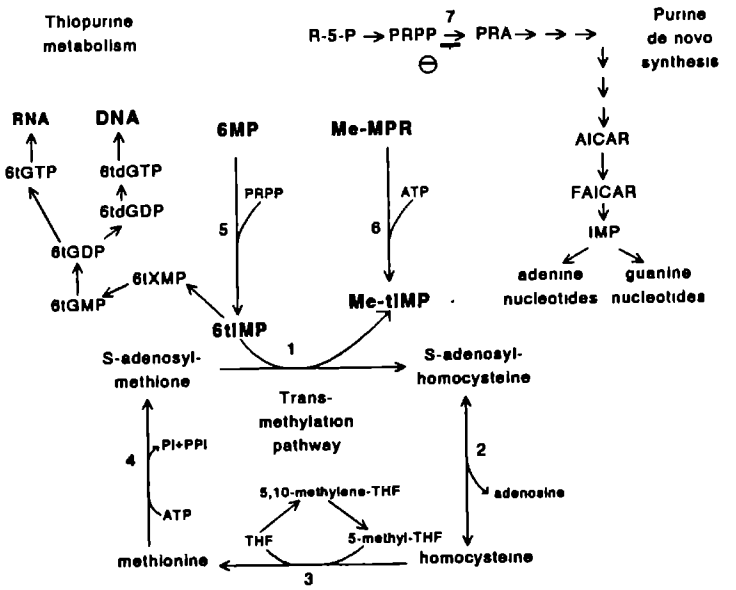
6-Mercaptopurine (6MP) and methylmercaptopurine ribonucleoside (Me-MPR) are purine antimetabolites which are both metabolized into Me-tIMP, a strong inhibitor of the purine de novo synthesis. Me-MPR is converted directly into Me-tIMP by adenosine kinase. 6MP is converted into thio-IMP (tIMP) and thereafter it is methylated into Me-tIMP by thiopurine methyltransferase, a S-adenosylmethionine (AdoMet) dependent conversion. AdoMet is formed out of methionine and ATP by methionine adenosyltransferase and is a universal methyldonor, involved in methylation of several macromolecules, e.g. DNA and RNA. Therefore, depletion of AdoMet could result in altered methylation state of these macromolecules, thereby affecting their functionality, leading to dysregulation of cellular processes and cytotoxicity. In this study the effects of 6MP and Me-MPR on AdoMet, S-adenosylhomocysteine (AdoHcy), homocysteine and methionine concentrations are determined. Both drugs cause a decrease of intracellular AdoMet concentrations and an increase of AdoHcy and methionine concentrations in Molt F4 human malignant lymphoblasts. The effects of both 6MP and Me-MPR can be ascribed to a reduced conversion of methionine into AdoMet, due to the ATP depletion induced by Me-tIMP formation. The observed decrease of the AdoMet/AdoHcy ratio by 6MP and Me-MPR affects the methylation state of the cells. This may result in dysregulation of cellular processes and may be an additional mechanism of cytotoxicity for 6MP and Me-MPR.

Introduction

6-Mercaptopurine (6MP), an analogue of the purine base hypoxanthine, is commonly used in the maintenance treatment of children with acute lymphoblastic leukemia (1). Two metabolic pathways contribute to 6MP cytotoxicity (Scheme 1). Both pathways are initiated by the conversion of 6MP into thio-IMP (tIMP) by the purine salvage enzyme hypoxanthine guanine phosphoribosyltransferase (2,3). Subsequently, tIMP can either be incorporated into DNA as thioguanine nucleotides (4-6), resulting in DNA damage (7-10) and consequently to delayed cytotoxicity (4,11,12), or tIMP can be methylated into methyl-tIMP (Me-tIMP). Me-tIMP is a strong inhibitor of purine de novo synthesis (13-15), inducing depletion of purine nucleotides. As a consequence, DNA and RNA synthesis become inhibited, resulting also in inhibition of cell growth and cytotoxicity (16,17).

Methylmercaptopurine ribonucleoside (Me-MPR), an other purine antimetabolite, exerts its

Scheme 1. 6-mercaptopurine metabolism and transmethylation pathway



(1) thiopurine methyltransferase (TPMT); (2) S-adenosylhomocysteine hydrolase; (3) methionine synthase; (4) methionine adenosyltransferase (MAT); (5) hypoxanthine-guanine phosphoribosyl-transferase (HGPRT); (6) adenosine kinase (AK); (7) phosphoribosyl pyrophosphate (PRPP) amidotransferase

cytotoxicity only after its conversion into Me-tIMP (16-19). Me-MPR is metabolized directly into Me-tIMP by adenosine kinase, in an ATP-dependent reaction (15,19-21).

Methylation of tIMP into Me-tIMP is a S-adenosylmethionine (AdoMet) dependent conversion, catalyzed by thiopurine methyltransferase (TPMT) (22-26). AdoMet serves as methyl donor for numerous methyltransferases, including those involved in methylation of proteins, phospholipids and nucleic acids, thereby affecting their function (27). These methyltransferases convert AdoMet into S-adenosylhomocysteine (AdoHcy), which is further converted into adenosine and homocysteine by AdoHcy hydrolase (28) (Scheme 1). Regeneration of AdoMet occurs by re-methylation of homocysteine into methionine by the vitamin B12 and methylenetetrahydrofolate reductase dependent methionine synthase. Methionine is the direct precursor for AdoMet synthesis (29,30). Alterations in the

transmethylation pathway have been found in most tumor cell lines and methionine-dependency is often observed in malignant cell lines (31).
In the present study the effects of various concentrations of 6MP and Me-MPR on cell growth, cell viability and ATP concentrations were determined in Molt F4 human malignant lymphoblasts. Because the conversion of 6MP into Me-tIMP consumes AdoMet, the concentrations of AdoMet, AdoHcy, homocysteine, methionine and of Me-tIMP were determined to establish possible effects of 6MP on the transmethylation pathway. The effects of Me-MPR on these parameters were also determined because Me-MPR is also converted into Me-tIMP, however, without the consumption of AdoMet.

Materials and Methods

Chemicals.
6MP, Me-MPR, S-adenosyl-L-methionine and S-adenosyl-homocysteine were obtained from Sigma Chemicals, USA.

Cells.
The experiments were performed with Molt F4 cells, a human T-cell acute lymphoblastic leukemia cell line. Conditions for cell culture have been previously described (32).

Experimental procedures.
Twentyfour h prior to the start of the experiments, logarithmically growing cells were seeded in a concentration of 0.2 x 10^6 cells per ml. At t = 0 6MP or Me-MPR were added as a single dose in a small volume (1/100). During the experiments 2 mM glutamine was supplemented every 24 h in order to prevent glutamine exhaustion of the medium (33). After incubation, the cells were harvested and counted with a coulter counter. Cell viability was determined by means of trypan blue exclusion. Cell growth was determined by counting the cells and correcting cell number for cell viability.

Determination of endogenous nucleotides and thionucleotides.
Endogenous nucleotides (di- and triphosphates) were determined in 3 x 10^6 viable cells, according to the method described before (34), and were measured at a wavelength of 254 nm. Nucleotide concentrations were expressed as pmoles/10^6 viable cells. Thionucleotides were extracted from 10^7 viable cells, according to the procedure described earlier (32). Thio-GMP was determined at 320 nm. Me-tIMP was measured at 290 nm. Concentrations were expressed as pmoles/10^6 viable cells.

Determination of AdoMet and AdoHcy.
AdoMet and AdoHcy concentrations were determined by means of HPLC in 10^7 viable cells according to the method described by Molloy et al. (35). The cell pellet was resuspended in 200 μl of 25 mM potassium phosphate buffer, pH 6.0 and proteins were precipitated by add-ing 20 μl 50 % trichloroacetic acid (TCA, BDH Chemicals Ltd, UK). The samples were kept on ice for 10 minutes and then centrifuged at 13,000 g for 2 minutes. Supernatants were collected and washed 3 times with 2 volumes of peroxide-free diethylether to remove TCA. The samples were flushed with nitrogen, while being on ice, and diluted with aquadest to an end volume of 200 μl. Hereafter, AdoMet and AdoHcy were separated on a Waters μBondapak C_{18} column (3.9 x 30 mm). Separation was performed with a gradient consisting of two buffers. Buffer A consisted of 25 mM potassiumphosphate (Sigma Chemicals, USA) and 10 mM heptansulphonic acid (Sigma Chemicals, USA), pH 3.2. Buffer B consisted of 12.5 mM potassiumphosphate, 50 % methanol and 10 mM heptansulphonic acid. The gradient was built up during consecutive time-points as follows: start 70 % buffer A + 30 % buffer B, 11 minutes 40 % A + 60 % B, 19 minutes 20 % A + 80 % B, 21 minutes 70 % A + 30 % B. One run lasted 30 minutes. The samples were analyzed by means of HPLC, consisting of a Kontron autosampler (MS1660, The Netherlands), a Spectra Physics pump (SP8800, Santa Clara, CA) and a uv spectrophotometer from ABI Separations (759A, The Netherlands). The data were processed with Spectra Physics Winner software (Santa Clara, CA).

Determination of extracellular homocysteine.
Total homocysteine concentrations were determined in 1 ml of culture medium after treatment of the cells with 6MP or Me-MPR. Samples were lyophilized overnight and thereafter resuspended in 0.2 ml aquadest. Subsequently total homocysteine was determined by means of HPLC, essentially according to the method described by Fiskerstrand et al. (36). A programmable sample processor (Gilson, model 232 BIO, Dilutor 401) was used for the automated homocysteine derivatization and sample injection. The pump (SP8800), the integrator (SP4400) and the fluorescence spectrophotometer (Linear Fluor LC 304) were from Spectra Physics (Santa Clara, CA).

Determination of intracellular methionine.
The intracellular methionine concentration was determined in 5 x 10^6 viable cells. The cells were centrifuged (5 minutes, 800 g) and the cell pellet was resuspended in 0.2 ml aquadest. The cells were sonified (3 x 10 sec, 15 Watt, Branson sonifier) and proteins were precipitated with 0.1 ml 18.8 % sulphosalicylic acid (Merck, Germany) with norleucine (600 μmol/l, Merck, Germany) as an internal standard. The suspension was kept on ice for 10 minutes. Subsequently, 0.1 ml buffer (pH 3.4) consisting of 0.2 M lithium citrate, 50 mM citric acid

(Merck, Germany), 200 mM lithium hydroxide (Fluka Chemie AG., Switz-erland), 10 mM phenol, 0.2 % thiodiglycol and 0.06 % HCl (Merck, Germany) was added and the suspension was centrifuged (4 minutes, 12,000 g). 0.2 ml aquadest were added to 0.2 ml of this suspension and 0.25 ml were used for determination of methionine. Methionine concentrations were determined using ion-exchange chromatography on an ami-noacid analyzer (Biotronik LC6001) according to the procedure from the manufacturer, with some modifications. To increase sensitivity of the method the aminoacids were derivatized with ortho-phtaldialdehyde (OPA, Sigma Chemicals, USA) and the methionine-ortho-phtal-dialdehyde adduct was quantified with a fluorescence detector (Jasco, model 821-FP).

Results

6MP induced a concentration dependent cytotoxicity in Molt F4 cells (results not shown).
Me-tIMP was detected in cells treated with 6MP (Table I). With 10 μM 6MP more Me-tIMP was formed than with 2 μM and with 10 μM 6MP the Me-tIMP concentration still increased after 24 h (Table I). The ATP concentration decreased as a result of treatment with 6MP (Table I). 10 μM 6MP induced a more severe depletion of ATP as compared to 2 μM 6MP. 6MP also decreased the concentration of AdoMet (Table II). Furthermore, treatment with 5 μM and 10 μM 6MP resulted in an increase of the AdoHcy concentration. The decrease of AdoMet and increase of AdoHcy resulted in a concentration-dependent decrease of the AdoMet/AdoHcy ratio.
After treatment with 6MP excretion of homocysteine was lower as compared to untreated cells (Table II). This was especially obvious 48 h after treatment. The intracellular homocysteine concentration in Molt F4 cells was below the detection limit. Intracellular *methionine concentrations increased after treatment with 6MP (Table II). With 2 μM 6MP some restoration of methionine concentration appeared after 48 h. With 5 μM and 10 μM 6MP*

Table I
ATP and Me-tIMP concentrations in Molt F4 cells treated with 2 or 10 μM 6MP. ATP concentration is expressed as pmol/10^6 viable cells; median and range (between brackets) of 9 independent experiments. Me-tIMP concentrations are expressed in pmoles/10^9 viable cells; median and range (between brackets) of 8 independent experiments.

	ATP			Me-tIMP	
time (h)	Control	2 μM 6MP	10 μM 6MP	2 μM 6MP	10 μM 6MP
24	5481 (3191-5792)	2516 (1886-3136)	1553 (963-2201)	119 (65-268)	338 (276-545)
48	4056 (2213-4532)	3084 (2118-3671)	2023 (1365-2533)	77 (25-301)	799 (291-868)

the methionine concentration increased further between 24 and 48 h.
The AdoMet/AdoHcy ratio appeared correlated with the ATP concentration, whereas the concentration of intracellular methionine appeared inversely correlated with ATP (Fig. 1).

Me-MPR is also converted into Me-tIMP, resulting in inhibition of purine de novo synthesis and ATP depletion. However, for this conversion no AdoMet is required. Therefore, the effects of Me-MPR on the inter-mediates of the methylation pathway were also determined. Me-MPR was also cytotoxic for Molt F4 cells, with maximal cytotoxicity at 0.5 μM Me-MPR (see chapter 5). Very high concentrations of Me-tIMP accumulated in cells treated with Me-MPR (Table III). After treatment with 0.2 μM Me-MPR the Me-tIMP concentration resembled that of cells, treated with 10 μM 6MP (Tables I and III). 0.2 μM Me-MPR resulted already in maximal inhibition of purine de novo synthesis during the first 24 h, as indicated by the depletion of the ATP pool (Table III). After 48 h, some restoration of the ATP pool occurred, especially in cells treated with 0.2 μM Me-MPR.
Me-MPR also induced a decrease of the AdoMet concentration and an increase of AdoHcy, resulting in a decreased AdoMet/AdoHcy ratio (Table II). Again, extracellular homocysteine

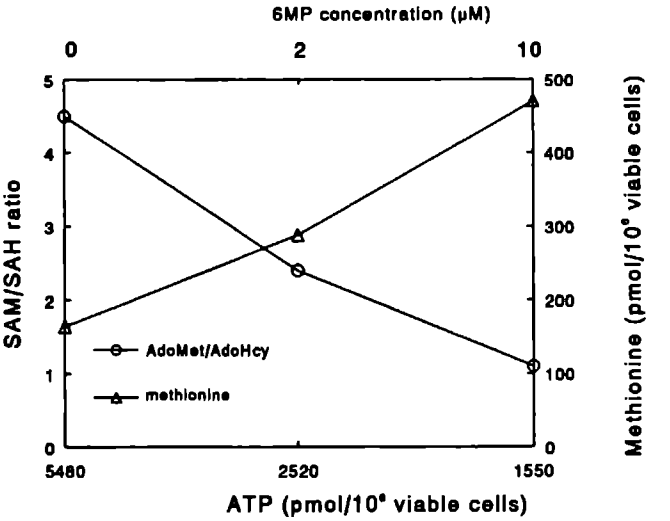


Fig. 1. Correlation between ATP concentration and methionine concentration and the AdoMet/AdoHcy ratio after treatment with 6MP for 24 h. The results of 1 experiment are shown. Similar results were obtained in 2 other experiments.

Table II

Intracellular concentrations of AdoMet, AdoHcy and methionine, and AdoMet/AdoHcy ratio, and extracellular concentrations of homocysteine after treatment with various concentrations 6MP or Me-MPR. The results of one experiment are shown. Similar results were obtained in 2 other experiments. AdoMet and AdoHcy concentrations are expressed as μmoles/10^7 viable cells. Methionine concentrations are expressed as pmol/10^6 viable cells. Homocysteine concentrations are expressed as μmoles/L.

	time (h)	control	2 μM 6MP	5 μM 6MP	10 μM 6MP	control	0.2 μM Me-MPR	0.5 μM Me-MPR	10 μM Me-MPR
AdoMet	24	5.0	2.4	2.2	2.3	5.2	2.7	2.6	2.2
AdoHcy		1.1	1.0	1.4	2.1	0.6	0.6	0.8	0.8
AdoMet/AdoHcy		4.5	2.4	1.6	1.1	8.7	4.5	3.3	2.8
AdoMet	48	3.2	2.7	2.4	2.5	4.8	3.4	2.4	2.2
AdoHcy		0.4	0.4	0.9	1.0	0.3	0.4	0.6	0.7
AdoMet/AdoHcy		8.0	6.8	2.7	2.5	16.0	8.5	4.0	3.1
Methionine	24	164	288	392	470	190	386	444	368
	48	88	154	570	786	92	168	336	458
Homocysteine	24	19.7	15.1	14.3	16.1	19.2	12.4	9.5	10.5
	48	33.6	16.5	12.7	16.3	34.9	14.0	11.2	11.2

Table III
ATP and Me-tIMP concentrations in Molt F4 cells treated with 0.2, 0.5 or 10 μM Me-MPR. ATP concentration is expressed as pmoles/10^6 viable cells; median and range (between brackets) of 3 experiments. Me-tIMP concentrations are expressed in pmoles/10^6 viable cells; median and range (between brackets) of 8 independent experiments.

	ATP				Me-tMP		
time (h)	Control	0.2 μM Me-MPR	0.5 μM Me-MPR	10 μM Me-MPR	0.2 μM Me-MPR	0.5 μM Me-MPR	10 μM Me-MPR
24	5481 (3191-5792)	1255 (1223-1458)	2063 (1468-2421)	1840 (1376-2307)	437 (386-540)	1450 (955-1601)	4718 (3608-7644)
48	4056 (2213-4532)	1970 (870-2163)	1866 (1268-2573)	2162 (1573-2504)	225 (223-225)	1301 (1014-1645)	3971 (2858-5682)

diminished as a result of treatment with Me-MPR as compared to untreated cells (Table II). This effect became obvious after 24 h and was more pronounced than after 6MP treatment. The methionine concentration also increased as a result of treatment with Me-MPR (TableII). The effects of Me-MPR on AdoMet, AdoHcy, methionine and homocysteine were comparable to those of 6MP.

Discussion

Extensive research has been performed to elucidate the mechanisms by which 6MP exerts its cytotoxic effects on tumor cells. Most attention has been focused on incorporation of 6MP into DNA as thioguanine nucleotides (4-6,11,12), whereas the methylation pathway for 6MP metabolism was considered to be a detoxifying process after low dose oral maintenance treatment and therefore negatively affecting therapy (37-39). However, our earlier studies indicated that the methylation route of 6MP, using concentrations of 2 μM and 10 μM 6MP, contributed to cytotoxicity by induction of inhibition of purine de novo syn-thesis (17,32,40). We now postulate a new mechanism by which formation of Me-tIMP from 6MP may also disturb various cellular processes, thereby inducing additional cytotoxicity.

Treatment of Molt F4 human malignant lymphoblasts with 6MP results in a depletion of AdoMet and an increase of AdoHcy, leading to a concentration-dependent decrease of the AdoMet/AdoHcy ratio (Table II). Theoretically, the depletion of AdoMet can be ascribed to two mechanisms. First, the 6MP metabolite tIMP is methylated by thiopurine methyl-transferase with AdoMet as methyldonor, thereby consuming AdoMet. However, with 2 μM 6MP less Me-tIMP is formed as compared with 10 μM 6MP (Table I), whereas AdoMet is equally depleted at these two concentrations of 6MP (Table II). Second, as a result of 6MP treatment and subsequent Me-tIMP formation, the purine de novo synthesis is inhibited, resulting in a depletion of ATP (Table I). ATP is also required as a substrate in the formation of AdoMet from methionine by methionine adenosyltransferase (Scheme 1). So depletion of ATP hampers the conversion of methionine into AdoMet. This mechanism could also account for the observed decrease of AdoMet and would explain the increase of the methionine concentrations (Table II).

To discriminate between both mechanisms we studied the effects of Me-MPR. Since Me-MPR is converted directly into Me-tIMP by adenosine kinase, no consumption of AdoMet occurs, whereas inhibition of purine de novo synthesis does occur. Furthermore, conversion of Me-MPR into Me-tIMP by adenosine kinase consumes ATP, and Me-MPR competitively inhibits adenosine kinase (chapter 6). Both mechanisms also contribute to the ATP depletion that results from Me-MPR treatment. In this way we could discriminate between the effects

of AdoMet consumption by methylation of tIMP into Me-tIMP versus reduced AdoMet synthesis as a result of ATP depletion. Overall, the effects of Me-MPR closely resemble those of 6MP. This demonstrates that the decreased AdoMet concentrations are caused by reduced synthesis due to decreased ATP levels.
The observed increase of the AdoHcy concentration (Table III) can not be explained by reduced synthesis of AdoMet. Conversion of AdoHcy into homocysteine by AdoHcy hydrolase is a reversible reaction, with its equilibrium strongly in favour of AdoHcy formation and an excess of adenosine induces accumulation of AdoHcy (28). Since Me-MPR is an analogue of adenosine, administration of Me-MPR could lead to the same effect, thereby inducing accumulation of AdoHcy and decrease of formation and excretion of homocysteine. This mechanism could also account for the effects of 6MP, since Me-MPR is also a metabolite of 6MP.

The observed effects of 6MP and Me-MPR on the transmethylation pathway may contribute to knowledge of 6MP cytotoxicity. First of all, AdoMet is the methyl donor for numerous methyltransferases, and is involved in methylation of e.g. nucleic acids, proteins and phospholipids (27), while AdoHcy is a potent inhibitor of most AdoMet-dependent transmethylases (28,41). So, the AdoMet/AdoHcy ratio is a major determinant of the cellular transmethylase activity (42). Compounds which influence pool sizes of AdoMet and AdoHcy and therefore the ratio of AdoMet/AdoHcy, may directly influence cellular methylation reactions, for instance methylation of DNA (31,43). This may induce differentiation in several tumor cell lines (44,45). Furthermore, methylation of DNA is thought to play a major role in gene regulation (46) and aberrant methylation patterns exist in DNA of tumor cells (31,47-49). Therefore, tumor cells may be especially sensitive to depletion of AdoMet.
In conclusion, both 6MP and Me-MPR may function as inhibitors of intracellular transmethylation reactions, due to a reduced AdoMet formation, thereby affecting cellular mechanisms on many different levels. Therefore, further research is warranted, possibly leading to a better understanding of the importance of the methylation route for 6MP cytotoxicity.

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CHAPTER 9

SUMMARY AND FUTURE DEVELOPMENTS

Summary and future developments

Summary

6MP was introduced as a drug against acute lymphoblastic leukemia some 40 years ago (1) and ever since, much research on its mechanisms of action has been performed. 6MP is metabolized by two pathways. The first one is conversion into thioguanine nucleotides and subsequent incorporation into RNA and DNA, thereby inducing DNA damage and leading to delayed cytotoxicity (2-17). The second one is formation of Me-tIMP and subsequent inhibition of purine de novo synthesis (PDNS). Inhibition of the PDNS leads to a depletion of purine nucleotide concentrations and results in inhibition of DNA synthesis (13,15,17-34). This leads to inhibition of cell growth and after prolonged exposure or exposure to high concentrations, to cell death. Adenine nucleotides function as energy donors for numerous metabolic processes (35) and guanine nucleotides are involved in processes such as protein synthesis, polyamine synthesis, microtubule assembly and intracellular signal transduction (36,37,38). So, depletion of purine nucleotides may induce cytotoxicity by many different mechanisms.

Incorporation of 6MP into DNA as thioguanine nucleotides is considered to be the main mechanism for 6MP cytotoxicity (3,5,7,12-15). In contrast, formation of Me-tIMP is considered to prevent cytotoxicity, since studies in leukemia patients receiving low dose oral 6MP reveal that an active thiopurine methylation pathway in red blood cells correlates with a poor prognosis (39). Furthermore, in patients with low or absent thiopurine methyltransferase (TPMT) activity, high concentrations of thioguanine nucleotides are detected in red blood cells and at the same time these patients are at risk for severe cytotoxic side-effects of the treatment (40,41). Other studies, however, indicate a role for Me-tIMP in 6MP cytotoxicity, since in phytohemaglutinin stimulated peripheral blood lymphocytes of subjects with genetically low TPMT activity, more 6MP is required for a 50 % inhibition of DNA synthesis, indicating that Me-tIMP may be of importance for 6MP cytotoxicity (42). Furthermore, Me-MPR, from which Me-tIMP is the sole active metabolite, has been used as an anticancer drug with pronounced cytotoxicity (27,28,43-47). These observations indicate the potential importance of Me-tIMP for 6MP and Me-MPR cytotoxicity.

The aim of this study was to obtain more knowledge on the importance of methylation for 6MP cytotoxicity. In previous years, investigators from our laboratory have extensively studied 6MP metabolism in vitro (16,17,48) and its pharmacokinetics in patients receiving oral 6MP treatment, as well as in dogs, goats and monkeys (32,49). Sensitive HPLC methods have been developed to determine both endogenous nucleotides and thiopurine nucleotides (50-52). These methods have provided us with the tools to study 6MP metabolism in

more detail in in vitro cell culture.
The experiments are performed in Molt F4, a T-cell ALL cell line. This is especially important, since leukemia cells are the ultimate target of the therapy and drug effects should be evaluated in cells closely resembling the target cells.

Chapter 1 reviews the knowledge concerning the mechanisms of 6MP cytotoxicity at the start of this study.
In chapter 2 the development of a sensitive micro-assay for measurement of the activity of IMPDH, the rate-limiting enzyme in purine interconversion and its inhibition by mycophenolic acid (MPA) are described. The specific activity of IMPDH in Molt F4 cells appears to be nearly twice as high as that in another leukemic cell line, KM_3, which is a non-B,non-T common ALL cell line. This indicates a high activity of purine metabolism in Molt F4 cells. MPA indeed inhibits the activity of IMPDH, as determined in pre-incubation experiments. This enables us to use MPA to study the importance of Me-tIMP formation for 6MP cytotoxicity in more detail, by preventing the conversion of tIMP into thioguanine nucleotides.
To determine the in vitro effects of MPA on cell growth and cell viability, Molt F4 cells are treated with several concentrations of MPA (chapter 3). MPA appears to be cytotoxic for Molt F4 cells. Cytotoxicity is concentration dependent. Furthermore, MPA induces a depletion of the guanine nucleotide concentration when administered to cells. Both cytotoxicity and depletion of intracellular guanine nucleotides is prevented by addition of guanosine to cells treated with MPA. Daily additions of guanosine are required to prevent cytotoxicity during the entire incubation period. In line with the high activity of purine salvage pathway and nucleotide metabolism, the intracellular guanine nucleotide concentrations rapidly decrease after an initial increase, when guanosine is added to the cells treated with MPA.
To study the importance of Me-tIMP formation for 6MP cytotoxicity more specifically, effects of combination of 6MP and MPA on cell viability and cell growth are studied (chapter 4). Based on the severe cytotoxicity of MPA (chapter 3), it is necessary to use low concentrations of MPA in the experiments where MPA is combined with 6MP.
The first indication for the importance of Me-tIMP for 6MP cytotoxicity is the observed synergism between 2 μM 6MP and 0.5 μM MPA. Since IMPDH is involved in the conversion of tIMP into thioguanine nucleotides, inhibition of this enzyme by MPA will decrease the formation of thioguanine nucleotides, leading to inhibition of incorporation of thioguanine nucleotides into RNA and DNA. Indeed, during the first 24 h after MPA and 6MP administration less tGMP was measured as compared to 6MP alone. Furthermore, the formation of Me-tIMP was increased after combination of 0.5 μM MPA and 2 μM 6MP. The observation that both cytotoxicity and Me-tIMP concentration increased as a result of

addition of MPA to 6MP treatment led to the conclusion that formation of Me-tIMP indeed may be of importance for the cytotoxicity of 6MP in lymphoblastic cells with an active PDNS.

Concentration dependent inhibition of PDNS by Me-tIMP is studied with increasing concentrations of Me-MPR (Chapter 5). Even as little as 0.1 μM Me-MPR results in maximal inhibition of PDNS, as reflected by the depletion of the purine nucleotides and by the increase of phosphoribosyl pyrophosphate (PRPP). The concentration of Me-tIMP which induces a maximal PRPP concentration and produces maximal effects on PDNS is 500 pmoles/10^6 viable cells. This concentration is also obtained in cells, treated with 10 μM 6MP, or with the combination of 2 μM 6MP and 0.5 μM MPA (chapter 4), indicating again that under these conditions inhibition of PDNS may indeed have a part in 6MP cytotoxicity.

The effects of inhibition of PDNS by Me-tIMP are examined in more detail by simultaneous prevention of purine nucleotide depletion. If Me-tIMP formation indeed exerts such a crucial effect, this should be eliminated by prevention of depletion of purine nucleotides, thereby decreasing 6MP cytotoxicity. Depletion of purine nucleotides can be prevented in two ways. First, by addition of AICAR, an intermediate of PDNS distal to the site of inhibition by Me-tIMP (Chapter 6). Second, by addition of intermediates of purine salvage, such as adenine, adenosine, inosine and guanosine (Chapter 7).

Addition of 50 μM AICAR to treatment with 2 μM 6MP indeed results in prevention of depletion of especially adenine nucleotides and a subsequent decrease of cytotoxicity (Chapter 6). However, as a result of addition of AICAR the concentrations of the intracellular metabolites of 6MP, being Me-tIMP, tIMP and tGMP, also decrease. Intracellular conversion of AICAR consumes PRPP. Since PRPP is also a cofactor for the conversion of 6MP into tIMP, addition of AICAR may diminish the incorporation of 6MP into the cells. Furthermore, cytotoxicity induced by 6MP partly results from incorporation of thioguanine nucleotides into DNA. So, the decrease of cytotoxicity after addition of AICAR can not exclusively be ascribed to prevention of depletion of purine nucleotides by circumventing the inhibition of PDNS.

Me-MPR is a thiopurine antimetabolite which sole metabolite is Me-tIMP. Therefore, by repeating the experiments with Me-MPR, instead of 6MP, the cytotoxic effects caused by inhibition of PDNS by Me-tIMP can be studied exclusively. Combination of Me-MPR and AICAR also results in decreased cytotoxicity, and restoration of purine, especially adenine nucleotide pools. Therefore these experiments stress the importance of Me-tIMP formation for cytotoxicity in Molt F4 cells. Furthermore, addition of AICAR hardly affects depletion of guanine nucleotide concentrations, but predominantly restores the adenine nucleotide concentrations, which indicates that depletion of adenine nucleotides, rather than of guanine nucleotides, is important for cytotoxicity of both drugs. Again, these experiments attribute to the idea that formation of Me-tIMP may induce cytotoxicity, especially in cells which

have a highly active PDNS.
This becomes also obvious from experiments where the intermediates of purine salvage adenosine, adenine, inosine and guanosine are used to prevent purine nucleotide depletion (Chapter 7). Adenosine, adenine and inosine are able to prevent depletion of adenine nucleotides, but not of guanine nucleotides in cells treated with 0.5 μM Me-MPR. At the same time combination of each of these purine salvage intermediates with 0.5 μM Me-MPR reduces cytotoxicity, thereby indicating that depletion of adenine nucleotides is the predominant cause of Me-tIMP induced cytotoxicity.
Addition of guanosine to treatment with 0.5 μM Me-MPR results in repletion of guanine nucleotides, associated with a more severe depletion of adenine nucleotides, especially early after addition of guanosine to treatment with 0.5 μM Me-MPR. At the same time, combination of 0.5 μM Me-MPR with guanosine also results in decreased cytotoxicity, indicating that depletion of guanine nucleotides also contributes to Me-tIMP cytotoxicity. Combination of guanosine with 10 μM Me-MPR results in a nearly complete depletion of adenine nucleotides, and an increase of cytotoxicity over that of 10 μM Me-MPR alone. The fact that depletion of ATP is so complete with the combination of a high concentration Me-MPR with guanosine can be ascribed to three processes. First, inhibition of PDNS by Me-MPR. Second, competitive inhibition of adenosine kinase by Me-MPR with respect to adenosine, and consumption of ATP during conversion of Me-MPR into Me-tIMP. Third, consumption of ATP by the conversion of guanosine into GTP, which concentration becomes extremely high upon addition of guanosine to the cells.
Thiopurines are methylated by thiopurine methyltransferase, with S-adenosylmethionine (AdoMet) as methyl-donor. As a result S-adenosylhomocysteine (AdoHcy) is formed. AdoMet is also the methyldonor for many other intracellular molecules, thereby regulating their functionality. Therefore, drugs which influence the intracellular methylation capacity may influence cellular processes on many levels. In chapter 8 the effects of treatment with either 6MP or Me-MPR on intermediates of the remethylation pathway, AdoMet, AdoHcy, homocysteine and methionine are examined. Me-MPR is converted directly into Me-tIMP by adenosine kinase with ATP as a co-substrate. This conversion is AdoMet independent, in contrast to Me-tIMP formation from 6MP. However, both 6MP and Me-MPR depleted the methylation capacity of the treated cells, reflected by a decreased AdoMet/AdoHcy ratio, as a result of a decrease of the AdoMet concentration and increase of AdoHcy. The decrease of AdoMet by 6MP can be ascribed to two mechanisms. First AdoMet is consumed for methylation of tIMP by thiopurine methyltransferase. Second, AdoMet formation appears to be inhibited as a consequence of ATP depletion resulting from inhibition of PDNS by Me-tIMP, since ATP is necessary for the conversion of methionine into AdoMet by methionine adenosyltransferase (MAT). This second mechanism also accounts for the increase of methionine observed as a result of 6MP treatment. Furthermore, Me-MPR also inhibits PDNS

via Me-tIMP, thereby inducing ATP depletion, so the same mechanism offers an explanation for the effects of Me-MPR on AdoMet and methionine concentrations. These results indicate that ATP depletion is the predominant cause for the decrease of AdoMet and the AdoMet/AdoHcy ratio.
The increase of AdoHcy, observed with both 6MP and Me-MPR can not be explained at this moment. It may be that Me-MPR directly or indirectly inhibits the conversion of AdoHcy into homocysteine by AdoHcy hydrolase, since Me-MPR is an analogue of adenosine, which is reported to exhibit feedback inhibition on this conversion.
So, both 6MP and Me-MPR reduce the AdoMet/AdoHcy ratio, thereby influencing the methylation capacity of the cells. The importance of the observed effects of 6MP and Me-MPR may be attributed to the fact that AdoMet is the methyl donor for many methyltransferases. The AdoMet/AdoHcy ratio is a major determinant of the methyltransferase activity, since AdoHcy is a potent inhibitor of several AdoMet-dependent transmethylases, and it is known that compounds which influence pool sizes, and especially the ratio of AdoMet/AdoHcy, may directly influence cellular methylation reactions, for instance methylation of DNA.

Future developments

The studies described in this thesis indicate that Me-tIMP formation attributes to cytotoxicity in Molt F4 cells, which exhibit a highly active purine de novo synthesis. This cytotoxicity may be attributed to inhibition of PDNS, and subsequent depletion of purine nucleotides, since prevention of this depletion results in prevention of cytotoxicity. However, the observed depletion of AdoMet, and the decrease of the AdoMet/AdoHcy may be an additional mechanism by which 6MP and Me-MPR exert their effects on cellular growth and cell viability.
Changes in AdoMet/AdoHcy ratio may directly influence methylation reactions (53-62). Methylation of DNA is thought to play a major role in gene regulation (63). It is repor-ted that an increase of the AdoHcy concentration induces a decrease of DNA methylation (56,64). Furthermore, administration of AdoMet leads to chemoprevention of rat liver carcinogenesis, indicating a role of DNA methylation in tumorprogression (59). Drugs, which influence AdoMet/AdoHcy ratio can induce differentiation in several tumor cell lines (57,65). Antimetabolites, such as 5-fluorouracil, 5-fluorodeoxyuridine and methotrexate are associated with profound DNA hypermethylation in Molt-4 cells (58). With this respect it is notable that treatment of HTB-54 cells with 6-thioguanine was associated with hypomethylation of DNA (58). At the same time it has been reported that in differentiating Friend erythroleukemia cells, DNA is hypomethylated (66), and that inhibition of DNA methyltransferase leads to induction of differentiation in several cell lines (67,68). So, the role of DNA methylation in tumor development is far from elucidated.

Besides the effects on methylation of DNA by a decrease of the AdoMet/AdoHcy ratio many other methylation processes may be affected by 6MP and Me-MPR. AdoMet is a methyl donor for many methylation processes, such as methylation of proteins, RNA and phospholipids, as well as for small molecules (69), thereby regulating their functionality. So, drugs which influence the transmethylation pathway may also exert effects on the function of all these molecules.

In conclusion, decrease of the AdoMet/AdoHcy ratio resulting from treatment with 6MP and Me-MPR may exert many effects in the cells, and may open a new field of research, possibly leading to a deeper understanding of the importance of methylation for 6MP cytotoxicity.

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Samenvatting

6-mercaptopurine (6MP) is ongeveer 40 jaar geleden geïntroduceerd als geneesmiddel tegen leukemie. Tegenwoordig wordt het gebruikt voor de onderhoudsbehandeling van kinderen met acute lymfatische leukemie.

6MP is een analoog van de purine base hypoxanthine. Dit wil zeggen dat 6MP qua structuur veel lijkt op hypoxanthine. Hypoxanthine heeft in cellen een aantal functies, het is onder andere een bouwsteen van DNA. 6MP vertoont zoveel overeenkomst met hypoxanthine dat het door cellen kan worden opgenomen en via dezelfde enzymen kan worden verwerkt als hypoxanthine.

6MP kan via twee mechanismen de dood van tumorcellen bewerkstelligen. Voor beide mechanismen moet 6MP eerst worden omgezet in thio-IMP. Dit thio-IMP kan dan door twee metabole routes worden omgezet:

Ten eerste kan thio-IMP via thio-GMP en thio-GDP worden omgezet in thio-GTP. Deze laatste verbinding kan worden ingebouwd in RNA en DNA van de tumorcellen. Inbouw van thio-GTP in DNA zorgt voor beschadiging van het DNA. Dit leidt tot celdood van de tumorcellen. Algemeen wordt aangenomen dat dit het belangrijkste mechanisme is waarop 6MP celdood (cytotoxiciteit) veroorzaakt.

Ten tweede kan thioIMP worden omgezet in methyl-thioIMP (Me-tIMP). Deze verbinding is een sterke remmer van de purine de novo synthese (PDNS), een metabole route waarbij in een aantal stappen nieuwe purine nucleotiden worden gevormd. Purine nucleotiden functioneren onder andere als bouwstenen voor RNA en DNA. Remming van de vorming van deze nucleotiden door Me-tIMP leidt tot een verminderde RNA en DNA-synthese. Aangezien tumorcellen vanwege hun hoge groeisnelheid een snelle RNA en DNA-synthese hebben, zorgt remming hiervan voor verlaging van celgroei en dood van de tumorcellen.

In dit proefschrift wordt het belang van de methylering (= de aanmaak van Me-tIMP) voor de cytotoxiciteit van 6MP onderzocht. Hiervoor zijn experimenten gedaan met 6MP en met metylmercaptopurine ribonucleoside (Me-MPR). Deze laatste verbinding wordt ook omgezet in Me-tIMP. Dit is meteen de enige metaboliet is van Me-MPR. Daarom kan met Me-MPR heel specifiek onderzoek worden gedaan naar de effecten van vorming van Me-tIMP. In het onderzoek is gebruik gemaakt van Molt F4 cellen, een T-cel acute lymfatische cellijn. Dit is wezenlijk, aangezien leukemiecellen het uiteindelijke doel van de therapie vormen.

Hoofdstuk 1 geeft een algemene inleiding over de kennis omtrent de cytotoxiciteit van 6MP en Me-MPR bij het begin van deze studie.

In hoofdstuk 2 wordt de ontwikkeling van een gevoelige micro-assay voor het bepalen van de activiteit van IMP-dehydrogenase (IMPDH) beschreven. IMPDH is het snelheidsbepalende enzym in de vorming van guanine nucleotiden. In Molt F4 blijkt het IMPDH twee

keer zo actief te zijn dan in een andere leukemische tumorcellijn, KM_3. Dit duidt op een actief purine metabolisme in Molt F4 cellen. IMPDH activiteit kan worden geremd met behulp van mycofenolzuur (mycophenolic acid, MPA), een specifieke remmer van het enzym.

In hoofdstuk 3 zijn de effecten van verschillende concentraties MPA op celgroei en celvitaliteit van Molt F4 cellen beschreven. Cytotoxiciteit van MPA is concentratie-afhankelijk. Verder leidt incubatie van de cellen met MPA tot een tekort aan guanine nucleotiden, doordat IMPDH wordt geremd. Door toevoeging van guanosine, dat via de 'purine salvage route' kan worden omgezet in guanine nucleotiden, kunnen zowel het tekort aan guanine nucleotiden als de cytotoxiciteit worden opgeheven.

In hoofdstuk 4 worden de effecten van de combinatie van MPA en 6MP op celgroei en celvitaliteit beschreven. Vanwege de hoge cytotoxiciteit van MPA (hoofdstuk 3), is voor de combinatie-experimenten een lage concentratie MPA gebruikt. Combinatie van 0.5 μM MPA met 2 μM 6MP leidt tot een synergistische werking van de cytotoxiciteit. Verder worden met deze combinatie minder thioguanine nucleotiden en meer Me-tIMP gevormd in vergelijking met 6MP alleen. Dat zowel cytotoxiciteit als Me-tIMP concentratie toenemen leidt tot de conclusie dat Me-tIMP vorming van belang is voor 6MP cytotoxiciteit in leukemische cellen met een actieve PDNS.

In hoofdstuk 5 is remming van de PDNS door Me-tIMP in meer detail onderzocht door gebruik te maken van oplopende concentraties Me-MPR. Na incubatie van de cellen met 0,2 μM Me-MPR blijkt er 500 pmol/10^6 cellen aan Me-tIMP te worden gevormd. Dit is voldoende om een maximale remming van de PDNS te veroorzaken, zoals blijkt uit het ontstane tekort aan purine nucleotiden en uit de toename van fosforibosylpyrofosfaat (PRPP). 500 pmol/10^6 cellen Me-tIMP wordt ook na incubaties met 10 μM 6MP, en met 2 μM 6MP in combinatie met MPA gemeten. Vorming van Me-tIMP kan dus ook zeker een rol spelen bij de cytotoxiciteit van 6MP.

De betekenis van remming van PDNS voor 6MP cytotoxiciteit is verder bestudeerd door middel van het voorkomen van het tekort aan purine nucleotiden. Dit kan op twee manieren. Allereerst kan dit door toevoeging van amidoimidazole carboxamide ribonucleoside (AICAR), een metaboliet die in de route van de PDNS voorkomt, na de plaats waar Me-tIMP deze route remt (hoofdstuk 6). Ten tweede kan het tekort aan purine nucleotiden worden voorkomen door gebruik te maken van metabolieten van de 'purine salvage route', zoals adenine, adenosine, inosine en guanosine (hoofdstuk 7). Door toevoeging van AICAR en van de laatstgenoemde metabolieten kan het tekort aan purine nucleotiden ten gevolge van de remming van de PDNS door Me-tIMP worden voorkomen.

Toevoeging van AICAR aan incubatie met 6MP blijkt vooral het tekort aan adenine nucleotiden te voorkomen (hoofdstuk 6). Tegelijkertijd neemt de cytotoxiciteit af. Aangezien AICAR echter ook zorgt voor een afname van de vorming van intracellulaire metabolieten

van 6MP (o.a. Me-tIMP), kan de verminderde cytotoxiciteit niet alleen worden toegeschreven aan het voorkomen van het tekort aan purine nucleotiden. Verder wordt een deel van de cytotoxiciteit van 6MP veroorzaakt door inbouw van thioguanine nucleotiden in DNA. Dit maakt interpretatie van de resultaten van de combinatie 6MP/AICAR moeilijk. Om die redenen zijn deze experimenten ook gedaan met Me-MPR in plaats van 6MP. Toevoeging van AICAR aan incubatie met Me-MPR voorkomt het tekort aan purine nucleotiden, vooral adenine nucleotiden. Dit leidt tot afname van de cytotoxiciteit. Dit wijst erop dat vooral het tekort aan adenine nucleotiden verantwoordelijk is voor de cytotoxiciteit van Me-tIMP.
Combinatie van adenine, adenosine of inosine met Me-MPR leidt ook tot een herstel van de adenine nucleotide concentratie en daarmee van de vitaliteit van de cellen (hoofdstuk 7). Opnieuw wordt hierdoor het belang van adenine nucleotiden tekort voor cytotoxiciteit aangetoond. Indien guanosine wordt toegevoegd aan incubatie met een hoge concentratie Me-MPR wordt daarentegen de cytotoxiciteit versterkt. Dit komt omdat bij de vorming van guanine nucleotiden, adenine nucleotiden worden verbruikt en deze daardoor nagenoeg volledig uitgeput raken in de cellen.
Vorming van Me-tIMP blijkt dus belangrijk te zijn voor cytotoxiciteit van 6MP. Voor vorming van Me-tIMP is S-adenosylmethionine (AdoMet) nodig als methyldonor. Bij deze reactie wordt het AdoMet omgezet in S-adenosylhomocysteine (AdoHcy). In hoofdstuk 8 worden de effecten van 6MP en Me-MPR op de AdoMet, AdoHcy en de methionine concentratie beschreven. 6MP blijkt een afname van AdoMet en een toename van AdoHcy en methionine te bewerkstelligen. De afname van AdoMet zou verklaard kunnen worden uit het verbruik van deze methyldonor voor de methylering van thio-IMP tot Me-tIMP. Echter ook Me-MPR zorgt voor een afname van AdoMet en een toename van AdoHcy en methionine, zodat verbruik van AdoMet voor de methylering van thio-IMP niet de enige oorzaak kan zijn voor de waargenomen effecten. AdoMet wordt gevormd uit methionine. Voor deze reactie is ATP nodig. Zowel 6MP als Me-MPR worden omgezet in Me-tIMP, wat leidt tot remming van de PDNS en dus tot een tekort aan ATP. De afname van AdoMet en de toename van methionine als gevolg van 6MP en Me-MPR kunnen daarom waarschijnlijk worden verklaard door de remming van de PDNS. AdoMet is een methyl-donor voor veel methyleringsreacties, onder andere van eiwitten, fosfolipiden, RNA en DNA. DNA methylering speelt een rol bij genexpressie. Methylering van eiwitten en fosfolipiden speelt een rol bij de functionaliteit van deze moleculen. Het is bekend dat geneesmiddelen die de concentratie AdoHcy verhogen, of de ratio tussen AdoMet en AdoHcy verlagen, de methyleringscapaciteit van cellen, en daarmee de methylering van bovengenoemde verbindingen kunnen veranderen, waardoor hun functionaliteit kan veranderen. Aangezien zowel 6MP als Me-MPR in staat blijken te zijn om AdoHcy te verhogen en de AdoMet/AdoHcy ratio te verlagen, is het mogelijk dat een deel van de cytotoxiciteit van deze cytostatica toegeschreven kan worden aan beïnvloeding van de AdoMet en AdoHcy concentraties.

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Eigenlijk is bijna iedereen op het laboratorium purines en pyrimidines wel op een of andere

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Curriculum vitae

Elisabet (Let) H. Stet werd geboren op 5 augustus 1961 te Tuindorp Oostzaan. 1979 behaalde zij het diploma gymnasium α aan de Rijksscholengemeenschap, te Brielle. Vervolgens deed zij in 1980 te Utrecht bij de "Stichting Aanvullend Onderwijs" de cursussen natuur- en scheikunde. Medio 1980 werd een aanvang gemaakt met de studie biologie, aan de Rijksuniversiteit Utrecht. Het doctoraal examen met als hoofdvakken Electronenmicroscopische Structuuranalyse (Moleculaire celbiologie), Experimentele Pathologie (Faculteit der Geneeskunde), en als bijvak Ontwikkelingsbiologie (Hubrecht Laboratorium, werkgroep Celinteractie) werd behaald in 1988. In de periode van 1 juli tot 1 oktober 1988 was zij werkzaam als analiste op het Hubrecht Laboratorium, onder leiding van prof. dr. E.J.J. van Zoelen.
Vanaf 16 april 1989 tot 16 april 1993 1994 was zij als wetenschappelijk medewerkster in dienst van de Nederlandse Kankerbestrijding werkzaam op het Laboratorium Kindergeneeskunde van het St. Radboudziekenhuis te Nijmegen, onder leiding van Prof. dr. Trijbels en dr. De Abreu. Van 16 april tot en met 31 december 1993 was zij werkzaam op hetzelfde laboratorium, in dienst van het Kinder Oncologisch centrum, Zuidoost Nederland.

Stellingen

behorende bij het proefschrift

The relevance of methylation for thiopurine cytotoxicity

In het openbaar te verdedigen
op 20 januari 1994
des namiddags te 1.30 uur precies

door

E.H. Stet

I

Uit de waarneming dat de cytotoxiciteit van 6-mercaptopurine in Molt F4 cellen toeneemt na remming van de synthese van thionucleotiden door mycophenolzuur, kan worden geconcludeerd dat methylering van 6-mercaptopurine in deze cellen een wezenlijke bijdrage levert aan de cytotoxiciteit van 6MP.
dit proefschrift

II

Depletie van adenine nucleotiden als gevolg van remming van de purine de novo synthese lijkt belangrijker voor cytotoxiciteit van thiopurines dan depletie van guanine nucleotiden.
dit proefschrift

III

De verlaging van de concentratie van S-adenosylmethionine die in Molt F4 cellen wordt veroorzaakt door zowel 6-mercaptopurine als methylmercaptopurine ribonucleoside kan leiden tot een verlaging van methylering van DNA en daarmee tot ontregeling van genexpressie en tot cytotoxiciteit.
dit proefschrift

IV

Het synergisme tussen 6-mercaptopurine en methotrexaat wordt toegeschreven aan verhoging van de phosphoribosyl pyrophosphaat concentratie als gevolg van de methotrexaatbehandeling, waardoor meer 6-mercaptopurine kan worden ingebouwd in de cellen[1]. Een additionele verhoging van de cytotoxiciteit kan veroorzaakt worden, doordat methotrexaat, evenals 6-mercaptopurine, de S-adenosylmethionine concentratie verlaagt[2,3].

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V

Het is bemoedigend dat na 40 jaar intensief onderzoek naar het werkingsmechanisme van 6-mercaptopurine een mogelijk nieuw mechanisme voor deregulering van cellulaire processen in de vorm van effecten op de methyleringscapaciteit van maligne cellen kan worden gevonden.
dit proefschrift

VI

De werking van 6-mercaptopurine op de verschillende metabole routes in de cel is dermate complex dat het buitengewoon moeilijk zal zijn om op grond van modelsystemen uitspraken te doen over de precieze mechanismen voor cytotoxiciteit van 6-mercaptopurine bij de behandeling van patiënten met acute lymfatische leukemie.

VII

Het is voor gemotiveerde en creatieve wetenschapsbeoefening onontbeerlijk om voldoende aandacht te besteden aan de beoefening van hobby's.

VIII

De billboard-slogan: "Roken, ook dat los je samen wel op" moet in analogie met rookreclames worden voorzien van de waarschuwing dat ook meeroken slecht is voor de gezondheid en hartklachten en kanker kan veroorzaken.

Nijmegen, 20 januari 1994 Let Stet